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E-cadherin mutations in cancer: impact on cell motility and signal transduction

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1 Introduction

1.1 Gastric cancer

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death worldwide (Parkin et al., 2001). The areas with the highest incidence rates are in Eastern Asia, the Andean regions of South America and Eastern Europe. Low rates are found in North America, North Europe, and most countries in Africa and South-eastern Asia (IARC Cancer Mondial Statistical Information System; http://www-dep.iarc.fr/). In the 1930s, gastric cancer was the most common cause of cancer-related death in the USA and Europe. A steady decline in the incidence and mortality rates of gastric carcinoma has been observed worldwide over the past several decades due to unplanned prevention. The decline took place following the popularization of refrigerators, which resulted in a decreased intake of salt for meat and fish preservation and the availability in many countries of fresh fruits and vegetables throughout the year (Palli, 2000).

Infection by *Helicobacter pylori* is prevalent in areas with high incidences of gastric cancers, and increases the risk of gastric tumours. Polymorphisms of proinflammatory cytokine genes have been shown to associate with risk of *H. pylori*-related gastric cancer (El-Omar et al., 2000). However, in some Asian countries, such as India and Thailand, incidences of gastric carcinomas are not high in spite of the high *H. pylori* infection rates, a phenomenon known as the "Asian Enigma" (Miwa et al., 2002). Possible explanations for this include host genetic factors, different virulence among strains of *H. pylori*, and dietary factors.

1.1.1 Tumour development and survival

Neoplasia of the stomach is largely composed of adenocarcinomas. The two main tumour sites of gastric adenocarcinoma are proximal (cardia) and distal (noncardia). Mesenchymal tumours, primary lymphomas, and carcinoid tumours can also arise in the stomach, but malignant tumours of these types occur less often.

Gastric cancer spreads by direct extension, metastasis or peritoneal dissemination. Early gastric cancer has a low incidence of vessel invasion and lymph-node metastasis. About 95 % of patients with early gastric cancer survive 5 years after

surgery. Patients with advanced gastric cancer have a 5-year survival rate of 10–30 %, depending on the depth of invasion (Roder et al., 1998). Most patients with advanced carcinoma already have lymph-node metastases at the time of diagnosis and therefore a poor survival rate. Apart from potentially curative surgery, chemotherapy as well as radiochemotherapy may be applied, but these do not cure the disease (Alberts et al., 2003). Thus, improvement of gastric cancer therapy will depend on novel therapeutic approaches.

1.1.2 Tumour classification

Several distinct morphologic, histologic, and biologic characteristics have been observed in gastric tumours. Multiple classification systems have been formulated in efforts to identify various subgroups with different biologic behaviour and prognostic indicators. According to the histologic growth pattern, gastric carcinoma is separated into diffuse and intestinal tumours (Lauren, 1965). Intestinal-type carcinomas have a glandular pattern and are usually accompanied by papillary or tubular formation or solid components. By contrast, the diffuse type consists of poorly cohesive cells diffusely infiltrating the gastric wall with little or no gland formation, and is associated with a worse prognosis. A special subgroup of this type is the so-called signet ring cell carcinoma in which a classical signet ring appearance is formed owing to the cell nucleus being pushed against the cell membrane, creating an expanded, globoid, optically clear cytoplasm (Watanabe et al., 1990). According to its cytological features, gastric carcinoma is classified into four grades. Adenocarcinoma is classified as grade I to III, signet ring cell carcinoma as grade III, and small cell and undifferentiated carcinoma as grade IV. The high grade (III-IV) comprise 65 % of all tumours and have a bad prognosis (Carriaga and Henson, 1995).

1.1.3 Gastric cancer predisposition

Most gastric carcinomas occur sporadically. Only 8-10 % have an inherited familial component (La Vecchia et al., 1992). Germline mutations in the gene encoding E-cadherin (CDH1) were first found in a large family from New Zealand in which diffuse-type gastric cancers took place at an early age (Guilford et al., 1998). These germline mutations lead to an autosomal dominant predisposition to gastric cancer, referred to as hereditary diffuse gastric carcinoma (HDGC) (Gayther et al., 1998; Guilford et al., 1999). Although E-cadherin germline mutations are very rare, their discovery provided useful information for clinicians to manage high-risk patients.

Gastric cancers, mainly of the intestinal type, can develop as part of the hereditary nonpolyposis colorectal cancer (HNPCC) syndrome, most cases of which are caused by germline mutations of mismatch repair genes hMLH1 or hMSH2, and are more prominently manifested in older generations of HNPCC patients. Patients with familial adenomatous polyposis, which is caused by germline mutations of APC, and Peutz-Jeghers syndrome also have increased risk for gastric cancer (Oberhuber and Stolte, 2000).

1.1.4 Molecular alterations in gastric cancer

Many studies in the past decade have clearly demonstrated that multiple genetic alterations – including changes in so-called tumour suppressor genes and oncogenes – are responsible for the development and progression of gastric cancer. Alterations, like mutation, overexpression or amplification, have been identified in specific genes that play important roles in diverse cellular functions such as cell adhesion, signal transduction, differentiation, development, gene transcription or DNA repair. Selected molecular changes ordered according to the stage of cancer development are depicted in Figure 1.1.

1.2 Cadherins

Adhesion between vertebrate cells is generally mediated by three types of adhesion junctions: tight junctions, adherens junctions (AJs), and desmosomes. Together they constitute the intercellular junctional complex, which has an important role in defining the physiological function of a cell.

Cadherins are Ca^{2+}-dependent cell adhesion molecules and the principal components of adherens junctions and desmosomes. Cadherins have a fundamental role in cell recognition during embryogenesis (Gumbiner, 2005; Takeichi, 1995; Tepass, 1999), during which specific expression patterns mark specific cell types and tissues. Virtually all cells that form solid tissues express cadherins, and cadherin expression is tightly regulated in the developing as well as in the adult organism. Downregulation or loss of cadherins in tumour cells often correlates with a more invasive phenotype (Gumbiner, 2005; Hajra and Fearon, 2002). In addition to its adhesive function, cadherins can modulate several signal transduction pathways by interacting with receptor tyrosine kinases, components of the Wnt signalling pathway and Rho-family GTPases.
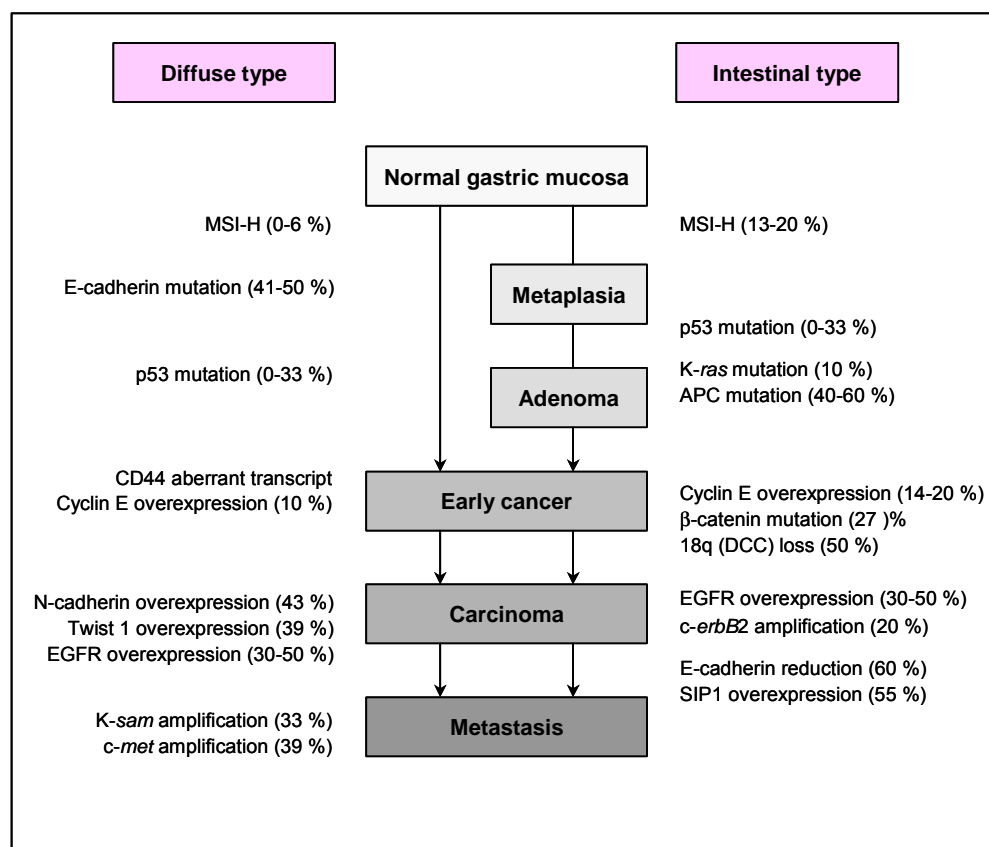
Figure 1.1: Genetic alterations in gastric cancer. Molecular changes differ between the two major types of gastric cancer: intestinal- and diffuse-type tumours. The alterations, such as mutation, overexpression or amplification, are ordered according to the stage of cancer development. Molecular changes in specific genes involved in cell adhesion, signal transduction, differentiation, development, gene transcription, and DNA repair have been identified. The percentages in parenthesis indicate the frequencies of the alterations observed when known. Abbreviations: APC, adenomatous polyposis coli; CD44, CD44 antigen; c-erbB2, v-erb-b2 erythroblastic leukaemia viral oncogene homologue 2; EGFR, epidermal growth factor receptor; c-met, met proto-oncogene (hepatocyte growth factor receptor); DCC, deleted in colon cancer; K-ras, v-Kiras2 Kirsten rat sarcoma viral oncogene homologue; K-sam, encodes fibroblast growth factor receptor 2; MSI-H, microsatellite instability-high; p53, tumour protein p53 (Li–Fraumeni syndrome); SIP1, SMAD-interacting protein 1; TWIST 1, twist homologue 1. (modified from Keller et al., 2005)

1.2.1 The cadherin superfamily

The evolutionarily diverse cadherin protein superfamily consists of approximately 80 members (Tepass et al., 2000). Cadherins are transmembrane glycoproteins with an N-terminal extracellular region, or ectodomain, that is followed by a transmembrane anchor and a C-terminal intracellular region. The cadherin superfamily is defined by the presence of characteristic ~110 amino-acid domains in the ectodomain and the sensitivity of their interactions to Ca^{2+}. The homology and number of these domains,

designated extracellular cadherin (EC) domains, determine the division of the cadherin superfamily into subfamilies (Nollet et al., 1999). These subgroups include classical cadherins (types I and II), desmosomal cadherins, protocadherins, and seven-pass transmembrane or Flamingo cadherins. In addition, cadherin family members have been identified that do not fit into a defined subfamily. Classical cadherins have five EC repeats, and type I (e.g. epithelial (E-) and neural (N-) cadherin) can be distinguished from type II (e.g. vascular-endothelial (VE-) cadherin) by the presence of a histidine, alanine, valine (HAV) tripeptide within the most N-terminal extracellular cadherin domain (EC1).

Calcium is essential for cadherin adhesive function and for protection against protease digestion (Grunwald et al., 1981; Takeichi, 1988). Structural studies have shown that calcium ions bind to specific residues in each EC domain to ensure its proper folding and to confer rigidity upon the extracellular domain (Koch et al., 1999).

1.2.2 Cadherin-mediated cell adhesion

Cells expressing different subtypes of cadherins segregate when mixed (Nose et al., 1988), and switches in the expression of particular cadherins correlate with the formation of specific tissues during embryonic development (Gumbiner, 2005). These observations led to the concept that cadherins function as homotypic cell adhesion molecules. The homophilic interaction between classical cadherins at the surface of adjacent cells is thought to be mediated by interactions between the HAV domains and between tryptophan residues and hydrophobic pockets in the most amino-terminal EC domains. How the remaining EC domains contribute to the stabilization of cell adhesion, and whether the cadherin molecules interact with each other in a zipper-like fashion, are matters of debate. Recent structural analysis by electron tomography indicates that individual cadherin molecules form groups and interact through their tips in a highly flexible manner (He et al., 2003)

Cell contacts initiated by the cadherin ectodomains strengthen over a time scale of minutes owing to rearrangements of the cytoskeleton near the developing junction (Chu et al., 2004). Moreover, during development adhesion must be closely linked to cell shape changes that enable morphogenetic movements as tissues remodel. Thus, the cytoplasmic side of cadherin-based cell junctions has an essential role in cell contact formation and in the interplay between adhesion and development.
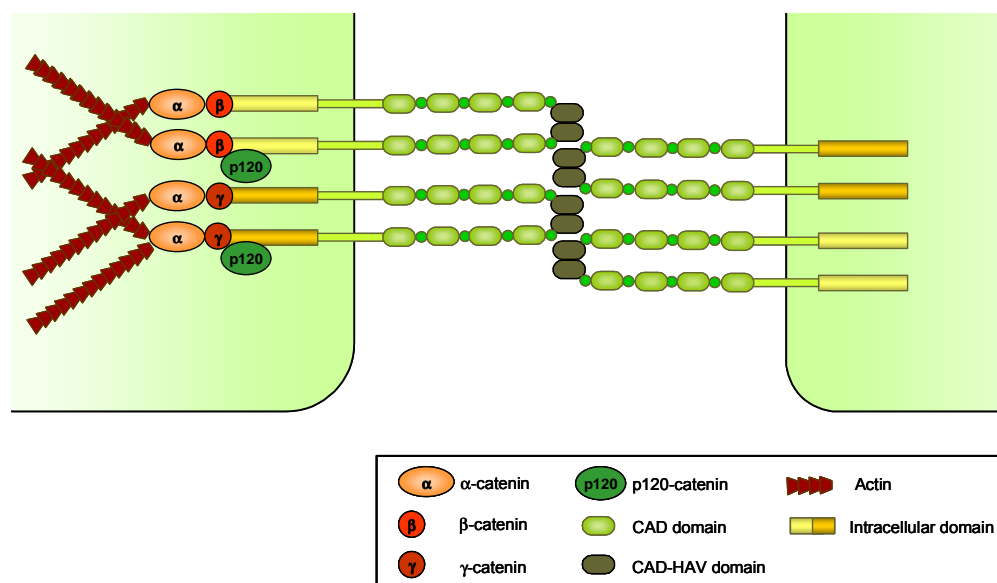
Figure 1.2: Model of cell-cell adhesion. Epithelial (E)-cadherin molecules that are expressed on the plasma membrane of adjacent cells probably interact in a zipper-like fashion. The most amino-terminal cadherin (CAD) domain on each E-cadherin molecule contains the histidine-alanine-valine (HAV) motif that is thought to interact with E-cadherins of adjacent cells. Recent evidence indicate that cadherin molecules interact through their tips in *cis* and in *trans* in a highly flexible manner. The cytoplasmic cell-adhesion complex (CCC), consisting of α-catenin, β-catenin, γ-catenin (plakoglobin) and p120 catenin, links E-cadherin homodimers to the actin cytoskeleton. (modified from Cavallaro & Christofori, 2004)

1.2.3   The cadherin-catenin complex

The intracellular domain of classical cadherins is the most highly conserved region of these molecules (Nollet et al., 1999) and interacts with various catenin proteins to form the cytoplasmic cell-adhesion complex (Figure 1.2). β-catenin and γ-catenin (also known as plakoglobin) bind to the same conserved site at the carboxyl termini of classical cadherins in a mutually exclusive way (Huber et al., 2001), whereas p120-catenin interacts with several sites in the cytoplasmic tail, including the juxtamembrane region. β-catenin and γ-catenin bind directly to α-catenin (Pokutta and Weis, 2000), which has a number of binding partners, including actin and several actin-binding proteins (Kobielak and Fuchs, 2004). On the basis of these binary interaction data, it was assumed for many years that α-catenin mediates a stable link between the cadherin–β-catenin complex and actin. Recent experiments have challenged this view and have suggested alternative roles for α-catenin in the junction (Drees et al., 2005; Yamada et al., 2005). The integrity of the so-called core complex including E-cadherin,

β-catenin and α-catenin is critical for the formation and/or maintenance of stable adhesion and its integrity is regulated by phosphorylation and dephosphorylation of β-catenin (Lilien et al., 2002).

1.2.4   E-cadherin in tumourigenesis

E-cadherin is a key player in inducing cell polarity and organizing an epithelium. In most cancers of epithelial origin, E-cadherin-mediated cell–cell adhesion is lost in the course of progression towards tumour malignancy. Although E-cadherin expression can still be found in differentiated tumours in patients, there is an inverse correlation between E-cadherin levels, tumour grade and patient mortality rates (Birchmeier and Behrens, 1994; Hirohashi, 1998). It has been proposed that the loss of E-cadherin-mediated cell–cell adhesion is a prerequisite for tumour-cell invasion and metastasis formation (Birchmeier and Behrens, 1994). Several groups demonstrated that re-establishing the functional cadherin complex resulted in a reversion from an invasive, mesenchymal phenotype to a benign, epithelial phenotype of cultured tumour cells (Birchmeier and Behrens, 1994; Vleminckx et al., 1991). Thus, the E-cadherin gene is also called a tumour or invasion suppressor gene.

The loss of E-cadherin function during tumour progression can be caused by various genetic or epigenetic mechanisms. In patients with diffuse gastric cancer and lobular breast cancer, and at a lower incidence in thyroid, bladder and gynaecological cancers, the E-cadherin gene is mutated (Strathdee, 2002). In most cases, E-cadherin expression is downregulated at the transcriptional level. The helix–loop–helix transcription factor E12/E47 and the zinc finger containing proteins Snail, Slug and SIP1 are transcriptional repressors that bind to E2 boxes in the promoter of the E-cadherin gene and repress its expression (Batlle et al., 2000; Comijn et al., 2001; Hajra et al., 2002; Perez-Moreno et al., 2001). As a direct consequence of transcriptional inactivation, the E-cadherin gene locus is epigenetically silenced by hypermethylation, leading to further downregulation of E-cadherin expression. Loss of heterozygosity (LOH) was also observed for the biallelic inactivation of E-cadherin (Scartozzi et al., 2004). Moreover it has been shown that *H. pylori* infection is associated with E-cadherin methylation and following downregulation (Terres et al., 1998).

1.2.5 E-cadherin mutations in gastric cancer

Becker and colleagues analysed the integrity of E-cadherin mRNA in gastric cancers and detected somatic E-cadherin mutations in 50 % of sporadic diffuse-type gastric carcinomas (Becker et al., 1994). These mutations are preferentially located in a mutational *hotspot* region within the second and third extracellular domain of E-cadherin (Gamboa-Dominguez et al., 2004). Gastric carcinoma-derived somatic E-cadherin mutations are mainly splice site and point mutations, affecting putative calcium binding sites (Handschuh et al., 1999). Expression of typical E-cadherin variants harbouring *in frame* deletions of exon 8 (*del 8*) or 9 (*del 9*) in MDA-MB-435S mammary carcinoma cells resulted in decreased adhesion and increased motility of the cells (Fuchs et al., 2005; Fuchs et al., 2002; Fuchs et al., 2004; Handschuh et al., 1999) and interfered with the proliferation-suppressive function of E-cadherin (Fricke et al., 2004). Thus, tumour-derived E-cadherin mutations have been shown to lead to a loss of function on one side and a gain of function on the other side, both of which are potentially relevant for tumour and metastasis formation. Moreover, wild-type E-cadherin was not detected in diffuse-type gastric carcinomas expressing mutant E-cadherin, providing further evidence for the function of E-cadherin as a tumour/ invasion suppressor.

Interestingly, the motility-promoting activity of mutant E-cadherin is sensitive to treatment with the EGFR-specific inhibitor Tyrphostin AG 1478 (Fuchs et al., 2002) identifying the EGFR signalling network as a mediator of the effect.

1.3 The ErbB family of receptor tyrosine kinases (RTK)

The ErbB receptors are expressed in various tissues of epithelial, mesenchymal and neuronal origin. They are involved in early embryonic development, in the renewal of stem cells in normal tissues such as skin, liver and gut and have a central role in the pathogenesis and progression of different carcinoma types (Normanno et al., 2001).

The ErbB family comprises four distinct receptors: EGFR (ErbB-1/Her1), ErbB-2 (Her2), ErbB-3 (Her3) and ErbB-4 (Her4) (Holbro et al., 2003). All family members are characterized by a modular structure consisting of an extracellular ligand-binding domain, a single transmembrane spanning helix and a cytoplasmic portion that contains a conserved but not equally functional tyrosine kinase domain. Only EGFR and ErbB-4 are fully functional in terms of ligand binding and kinase activity. ErbB-3 has impaired kinase activity (Guy et al., 1994) and relies on the kinase activity of its

heterodimerization partners for activation. ErbB receptors are activated by binding to growth factors of the EGF-family. These factors are either produced by the same cells that express ErbB receptors (autocrine secretion) or by surrounding cells (paracrine secretion). With respect to ErbB receptor binding, the EGF family of ligands can be divided into three groups (Yarden and Sliwkowski, 2001). The first includes EGF, transforming growth factor-α (TGF-α) and amphiregulin (AR), which bind specifically to EGFR; the second includes betacellulin (BTC), heparin-binding growth factor (HB-EGF) and epiregulin (EPR), which show dual specificity, binding both EGFR and ErbB-4; the third group is composed of the neuregulins (NRGs) and form two subgroups based on their capacity to bind ErbB-3 and ErbB-4 (NRG1 and NRG2) or only ErbB-4 (NRG3 and NRG4). None of the known ligands binds ErbB-2 (Figure 1.3).
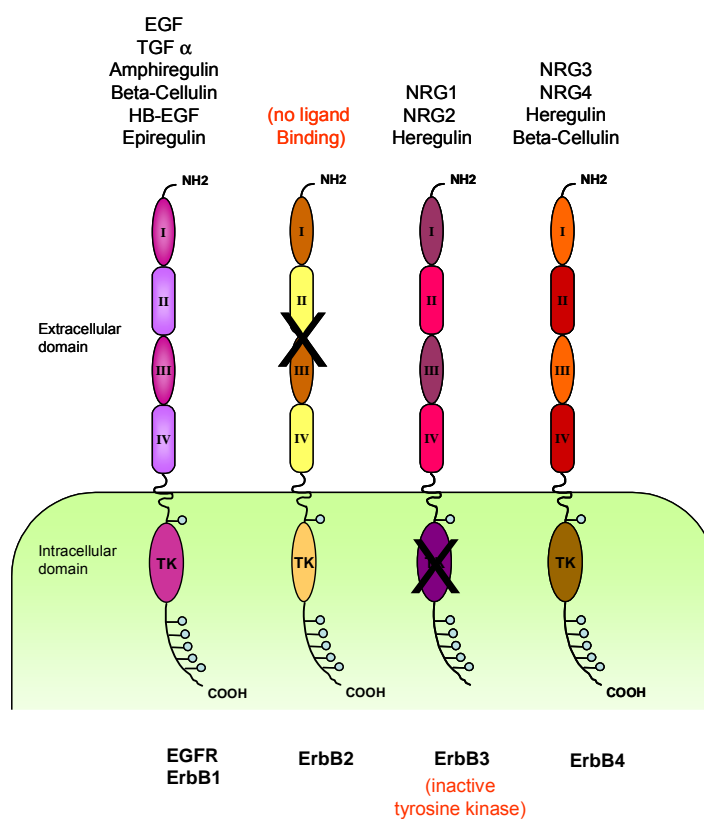
Figure 1.3: The ErbB receptor family. Each member of the ErbB receptor family consists of an extracellular ligand-binding domain comprising four subdomains (I-IV), a transmembrane spanning helix and a cytoplasmic portion harbouring a tyrosine kinase domain (TK). Only EGFR and ErbB-4 are fully functional in terms of ligand binding and kinase activity. ErbB3 has impaired kinase activity and no ErbB2 ligand has been detected so far. (modified from Hynes andLane 2005)

1.3.1 Receptor activation

Ligand binding to the extracellular domain of ErbB receptors induces the formation of homo- and heterodimeric receptor complexes between members of the ErbB family (Olayioye et al., 2000). The intrinsic EGFR kinase catalyses the autophosphorylation of key tyrosine residues in the cytoplasmic tail of each receptor monomer in a *trans* manner (Honegger et al., 1990). These phosphorylated residues serve as docking sites for proteins containing Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains. The recruitment of these adaptor and signalling molecules results in the activation of several intracellular signalling pathways downstream of the receptor leading to cell proliferation, motility and survival.

Recently, new insights in the process of ligand-induced receptor dimerization were provided by studies on the crystal structures of EGFR, ErbB-2 and ErbB-3's extracellular domains (Burgess et al., 2003; Cho and Leahy, 2002; Cho et al., 2003; Garrett et al., 2003; Garrett et al., 2002; Ogiso et al., 2002). The extracellular portion of each ErbB receptor consists of four subdomains (I-IV). Subdomains I and III have a beta helical fold and are important for ligand binding. A putative dimerization loop in subdomain II promotes direct receptor-receptor interaction which shows that EGFR dimerization is not mediated by the ligand itself. In the crystal structure of the extracellular domain of EGFR bound to EGF, the dimerization loop protrudes from EGFR and mediates interaction with another EGFR monomer leading to the formation of a dimer composed of two receptor/ligand complexes (Garrett et al., 2002; Ogiso et al., 2002). In contrast, the so-called tethered structure of inactive EGFR or ErbB-3 is characterized by intramolecular interactions between domain II and IV which block the domain II dimerization interface (Cho and Leahy, 2002; Ferguson et al., 2003).

The structure of ErbB-2 extracellular region differs significantly from EGFR and ErbB-3. In the absence of a ligand, ErbB-2 has a conformation that resembles the ligand-activated state with a protruding dimerization loop. In this conformation, domains I and III are very close and this interaction makes ligand binding impossible, explaining why no ErbB-2 ligand has been detected so far (Garrett et al., 2003). The structure of ErbB-2 is consistent with the data indicating that the receptor is the preferred dimerization partner for the other ErbBs, as it is permanently available for interaction with another ligand-bound receptor.
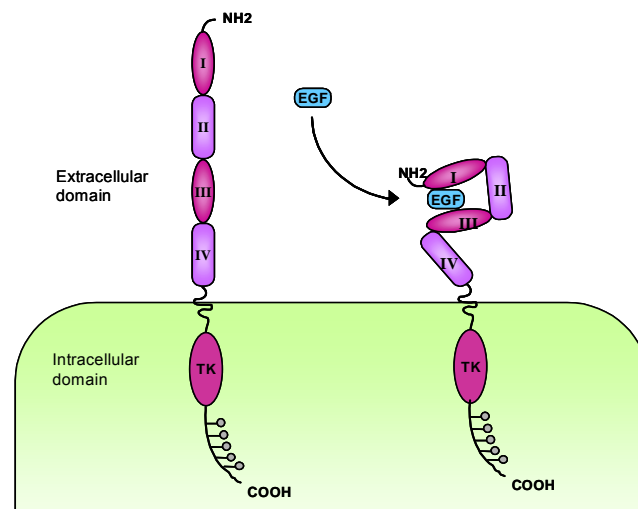
Figure 1.4: EGFR structure. The extracellular portion of EGFR is composed of four subdomains (I-IV). The domains I, II and III form a ligand-binding pocket, where a ligand is bound between domains I and III. (modified from Zandi et al., 2007)

1.3.2 Signalling pathways activated by EGFR

The functional diversity of proteins that interact with EGFR after its autophosphorylation results in the simultaneous activation of multiple pathways. These pathways include those that involve Ras activation leading to various MAP kinases (Alroy and Yarden, 1997), other small GTPases such as Rho and Rac, multiple STAT (signal transducer and activator of transcription) isoforms (David et al., 1996), PLCγ (phospholipase C-γ) (Thomas et al., 2003) and its downstream calcium and PKC-mediated cascades, heterotrimeric G proteins, as well as other pathways including the phospholipid-directed enzymes PI3 kinase (phosphatidylinositol 3'-OH kinase) and the cytoplasmic tyrosine kinase Src (Hackel et al., 1999).

The Ras-Raf-MEK-ERK pathway is one of the best characterized EGFR effector pathways. The key player in EGF-dependent Ras activation is the adaptor protein Grb2 (Lowenstein et al., 1992). Grb2 is constitutively bound to the Ras exchange factor Sos and is localized to the cytosol. Following activation of EGFR, the SH2 domain of Grb2 can bind to EGFR directly or indirectly by binding to EGFR-associated, tyrosine phosphorylated Shc (Sasaoka et al., 1994). Relocation of the Grb2/Sos complex to the receptor at the plasma membrane facilitates the interaction of membrane-bound Ras with Sos, resulting in the exchange of Ras-bound GDP for GTP and hence in Ras

activation (Aronheim et al., 1994). Both Grb2 and Shc play important roles in the activation of other EGFR-dependent pathways. Grb2 contains two SH2 domains and one SH3 domain, which enables it to interact with tyrosine-phosphorylated motifs as well as with proline-rich regions of other proteins. Shc can associate with specific tyrosine-phosphorylated sequences via its SH2 and PTB domain and, being itself phosphorylated on tyrosine by activated receptors and cytosolic tyrosine kinases, serve in turn as a binding partner for SH2-containing proteins.

The cytosolic tyrosine kinase Src has long been implicated in EGFR signal transduction. Overexpression of Src strongly enhances EGF-mediated proliferation and transformation in epithelial cells (Luttrell et al., 1988; Maa et al., 1995). Conversely, inhibition of Src activity can block EGF-dependent DNA synthesis (Barone and Courtneidge, 1995; Roche et al., 1995) and reverse the transformed phenotype of EGFR- or ErbB2-overexpressing cells (Karni et al., 1999). However, it is still not clear whether Src is a signal transducer downstream of EGFR or a contributor to EGFR activation.
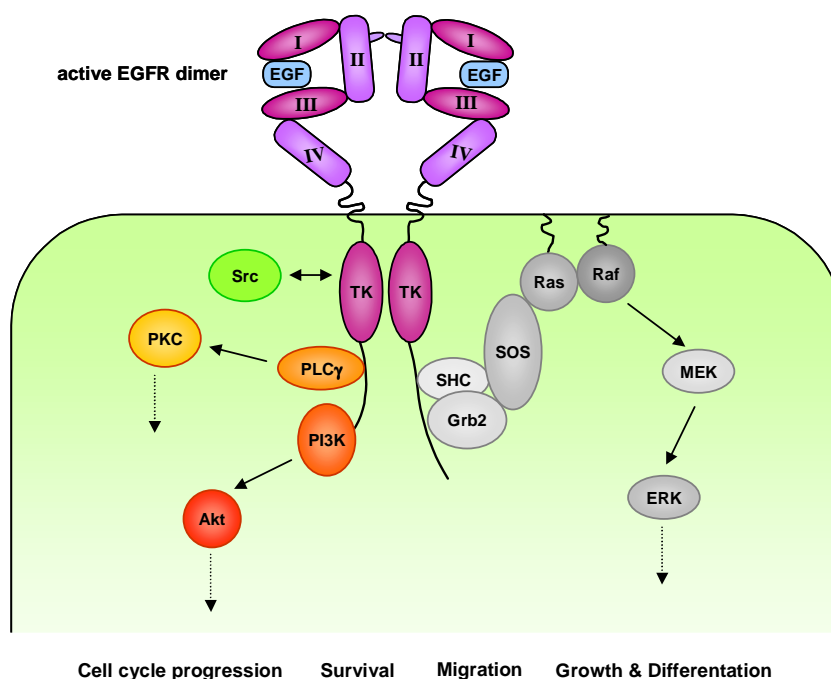
Figure 1.5: EGFR signalling. Binding of ligand to EGFR leads to receptor dimerization, autophosphorylation and activation of several downstream signalling pathways. Only selected signalling molecules are presented. (modified from Zandi et al., 2007)

1.3.3 Compartmentalization of EGFR signalling

The cellular microenvironment plays a critical role in the control of signalling. Beside the plasma membrane, activated receptors are also rapidly relocated inside the cell and often transferred to locally pre-assembled signalling complexes, the so-called 'signalosomes'. These structures are usually localized to subcellular compartments like membrane microdomains, endosomal vesicles, ribosomes etc (Szymkiewicz et al., 2004).

On the cell surface, EGFR can be found in several distinct microdomains like membrane rafts and caveolae. Rafts are small (10-20 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes (Pike, 2005). Caveolae are flask-shaped invaginations, also enriched in cholesterol but surrounded by a coat of the structural protein caveolin-1. The estimated numbers for the fraction of EGFR in these microdomains are around 40-60 % (Mineo et al., 1999) and EGFR appears to be more enriched in rafts (40 %) than in caveolae (5-10 %) (Ringerike et al., 2002). EGFR activation induces migration of the receptor from rafts/caveolae to the bulk membrane component and the clustering of EGFR complexes into clathrin-coated pits that are subsequently internalized (Beguinot et al., 1984). Cholesterol depletion results in disruption of rafts/caveolae which leads in turn to an increase in EGFR dimerization and kinase activity as well as the enhancement of downstream signalling pathways (Pike, 2005). Thus, rafts/caveolae have been suggested to be inhibitory for signalling while they serve at the same time to gather a variety of functionally linked proteins (Couet et al., 1997; Lim and Yin, 2005).

1.3.4 Attenuation of EGFR signalling

Inactivation of EGFR can be mediated either by dephosphorylation of key residues by phosphotyrosine phosphatases or receptor downregulation which is the most prominent regulator of EGFR signal attenuation. Receptor downregulation involves the internalization and degradation of the activated receptor in the lysosomes (Lipkowitz, 2003; Shtiegman and Yarden, 2003). Although ErbB receptors are primarily localized to the basolateral cell surface in various human epithelial tissues (Playford et al., 1996), they constantly shuttle and recycle through the cell. An interesting aspect of ErbB receptors is that only EGFR appears to undergo ligand-induced internalization. The rate of EGFR endocytosis is increased 5-10 fold following ligand activation, whereas the internalization rate of ErbB2-4 appears to be similar in both the empty and the

occupied states (Waterman et al., 1998; Worthylake et al., 1999). Functionally, this means that only EGFR displays acute, ligand-induced receptor degradation. To compensate the lack of induced degradation, the other ErbB family members show a more rapid rate of constitutive turnover. For example, the half-life of ErbB2 in CHO cells is only 3.5 h, compared to 10 h for the EGFR (Citri et al., 2002). After growth factor binding, EGFR homo- and heterodimers are targeted to clathrin-coated pits. Intracellular routing of EGF receptors to the mildly acidic early endosome is followed by the decision whether the ligand-receptor complex dissociates, with the receptor being recycled or not. Complexes which remain stable under these conditions, such as the EGF/EGFR complex, are directed to multi-vesicular bodies and late endosomes for degradation, thus reducing the cell surface content of the receptor and its signal capacity. In contrast, interaction of TGF-α and EGFR is pH sensitive. This results in complex dissociation in the endosome and recycling to the cell membrane of the receptor where it will be available for a new cycle of activation by ligands (Waterman et al., 1998).

1.3.5 Crosstalk between EGFR and E-cadherin

Numerous studies indicate that diverse classes of RTKs, including EGFR, can downregulate and inhibit E-cadherin-dependent adhesion when they induce EMT (epithelial-to-mesenchyme transition) (Thiery 2002). EGFR activation provokes tyrosine phosphorylation of components of the cell adhesion complex, resulting in disruption of cell adhesion and endocytosis of E-cadherin (Bryant and Stow, 2004). In turn, functional adhesion junctions can affect the activity and localization of RTKs. EGFR has been shown to colocalize with E-cadherin (Hoschuetzky et al., 1994). Recent results demonstrate that E-cadherin-mediated adhesion inhibits ligand-dependent activation of EGFR, also pointing to a crosstalk between EGFR and E-cadherin with potential relevance for tumour and metastasis formation (Qian et al., 2004; Takahashi and Suzuki, 1996). Complex formation of E-cadherin with EGFR and other RTKs like c-Met or ErbB2 was shown to be mediated through the extracellular domain of E-cadherin, thereby decreasing receptor motility and ligand binding affinity (Qian et al., 2004). In contrast, E-cadherin has been found to activate transiently EGFR when cell-cell contacts were formed by switching from low-calcium conditions to high calcium (Pece and Gutkind, 2000). Taken together, these findings imply that the regulation of RTKs and E-cadherin is bidirectional.

1.3.6 EGFR and cancer

As EGFR is involved in the regulation of cellular processes like proliferation, motility and survival, disruption of the tight regulation of the EGFR-ligand system can lead to neoplastic transformation. In fact, dysregulated EGFR has frequently been found in various forms of human cancers (Arteaga, 2002; Prenzel et al., 2001). Mechanisms by which EGFR regulation is abrogated include increased production of ligands, increased levels of EGFR protein, EGFR mutations giving rise to constitutively active variants, defective downregulation of EGFR and crosstalk with heterologous receptor systems (Zandi et al., 2007). Patients with cancer whose tumours have alterations in EGFR tend to have a more aggressive disease that predict a poor clinical outcome (Hynes and Lane, 2005).

The ErbB receptors are targets for selective anti-cancer therapies. Two major classes of ErbB antagonists have been successfully tested in phase 3 trials and are now in clinical use: ectodomain-binding monoclonal antibodies and small-molecule tyrosine kinase inhibitors (TKIs). Anti-EGFR monoclonal antibodies bind to the extracellular domain of EGFR when it is in the inactive conformation, compete for receptor binding by occluding the ligand-binding region, and thereby block the ligand-induced EGFR tyrosine kinase activation. TKIs compete reversibly with ATP in the tyrosine kinase domain and inhibit EGFR autophosphorylation and downstream signalling. Two anti-EGFR monoclonal antibodies (cetuximab/Erbitux[®] and panitumumab/Vectibix™) and two TKIs (gefitinib/Iressa[®] and erlotinib/Tarceva[®]) have been approved in several countries for the treatment of metastatic non-small-cell lung cancer, squamous-cell carcinoma of the head and neck, colorectal cancer, or pancreatic cancer. In addition, an anti-ErbB2 monoclonal antibody (trastuzumab/Herceptin[®]) has been approved for patients with invasive breast cancers that overexpress ErbB2. Trastuzumab binds to the extracellular juxtamembrane domain of ErbB2 and inhibits the proliferation and survival of ErbB2-dependent tumours.

1.4 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) have been implicated in many different aspects of development and disease. MMPs appear to regulate cellular behaviour through pericellular proteolysis (Werb, 1997). Proteases are required for the ability of cells to degrade components of the extracellular matrix (ECM) and to interact with and influence other cells. Proteases are also important for the processing of proteins that lead to their activation, deactivation and removal. The MMPs are secreted proteases that function in the extracellular matrix to maintain homeostasis as well as to influence matrix reorganization.

The MMPs are synthesized as inactive zymogens (pro-MMPs). They are kept inactive by an interaction between a cystein-sulphydryl group in the propeptide domain and the zinc ion bound to the catalytic domain. The activation of MMPs requires proteolytic removal of the propeptide domain (Sternlicht and Werb, 2001). Most of the MMPs are activated outside the cell by other MMPs or serine proteinases and MMP activity is tightly controlled by endogenous inhibitors. There are eight distinct structural classes of MMPs: five are secreted and three are membrane-type MMPs (MT-MMPs).

Historically, MMPs were thought to predominantly degrade structural components of the ECM, thereby facilitating cell migration. Cleavage of ECM proteins by MMPs also modulates cellular signalling and function because receptors for ECM components like the integrins are affected. In addition, MMPs participate in the release of membrane-bound precursor forms of many growth factors. Cell adhesion molecules and growth factor receptors are also MMP substrates. Cleavage of E-cadherin and CD44 results in the release of fragments of the extracellular domains and in increased invasive behaviour (Kajita et al., 2001; Noe et al., 2001).

1.4.1   Reciprocal interaction between E-cadherin and MMPs

There is evidence that E-cadherin plays a role in the acquisition of the invasive phenotype via regulation of MMP expression. This provides a functional link between cell-matrix adhesion and pericellular proteolytic potential. Overexpression of E-cadherin in a highly invasive bronchial tumour cell line decreased invasiveness and reduced expression of MMP-1, MMP-3, MMP-9 and MT1-MMP (Nawrocki-Raby et al., 2003). Consistent with decreased MMP expression as a result of E-cadherin overexpression, loss of E-cadherin mediated cell-cell adhesion has been shown to increase MMP

levels. Fuchs and colleagues demonstrated that expression of gastric carcinoma-derived mutant E-cadherin in MDA-MB-435S cells caused elevated levels of MMP-3 as compared to *wt* transfectants (Fuchs et al., 2005). By application of NNGH, a synthetic inhibitor of MMPs, or siRNA directed against MMP-3, cell motility stimulated by mutant E-cadherin was downregulated. These results point to a functional link between MMP-3 and E-cadherin and reveal a novel effect of mutant E-cadherin: the upregulation of the proteolytic function of tumour cells by enhanced secretion of MMP-3.

E-cadherin regulates MMP expression. In turn, MMPs have been shown to modulate the junctional localization of E-cadherin by proteolytic modification of the E-cadherin ectodomain, suggesting reciprocal interactions between MMPs and E-cadherin. Overexpression of active MMP-3 in breast cancer cells was sufficient to induce EMT by promoting E-cadherin ectodomain shedding, leading to the subsequent disappearance of cadherin and catenin from cell-cell junctions (Xian et al., 2005).

1.4.2   MMPs and cancer

The expression and activity of MMPs are increased in almost every type of human cancer, and this correlates with advanced tumour stage, increased invasion and metastasis, and shortened survival. Unlike classical oncogenes, MMPs are not upregulated by gene amplification or activating mutations. The increased MMP expression is probably due to transcriptional changes rather than genetic alterations.

Pharmaceutical inhibitors of MMPs have been developed but clinical trials provided disappointing results. One explanation might be that there are six fundamental alterations in cell physiology underlying cancer progression: self-support in growth signals, insensitivity to growth-inhibitory signals, escape from apoptosis, infinite replication, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). Originally, MMPs were considered to be important almost exclusively for the late steps of tumour progression, i.e. invasion and metastasis. The first clinical trials were designed on this assumption. Recent studies document that MMPs are involved in several other steps of cancer development. To better target MMPs for cancer treatment, an understanding of their many functions is needed (Overall and Kleinfeld, 2006).

1.5 Aim of this study

The aim of the present study was to understand the molecular mechanism of how gastric carcinoma-derived E-cadherin mutations enhance cell motility.

Mutation of E-cadherin and aberrant EGFR signalling have been associated with gastric cancer. Tumour-derived E-cadherin mutations cause increased motility of the cells by an unknown mechanism. Motility enhancement by mutant E-cadherin is sensitive to treatment with an EGFR tyrosine kinase inhibitor or a synthetic MMP-3 inhibitor, identifying both molecules as mediators of the effect. However, the molecular mechanism linking E-cadherin mutations, the EGFR signalling pathway and MMP-3 remained unresolved.

Therefore, the present study attends to the following aspects:

- The crosstalk between mutant E-cadherin and EGFR was examined. In particular, the hypothesis that EGFR activation might be observed in the presence of somatic E-cadherin mutations was specified in a cell culture model. A panel of phospho-epitope specific anti-EGFR antibodies was used to investigate phosphorylation at different tyrosine residues in the C-terminal domain of EGFR after ligand binding.

- The modulation of EGFR downstream signalling by enhanced receptor activation in the presence of mutant E-cadherin was analysed. The interaction of EGFR and the adapter proteins Grb2 and Shc, which are recruited to the receptor after its activation, was explored by immunoprecipitation. In addition, activation of the small GTPase Ras was analysed by Raf1-RBD pull-down assays.

- EGFR and E-cadherin colocalize in a multicomponent complex and the extracellular domain of E-cadherin is critical for this interaction. By using immunoprecipitation analysis, the question whether mutant E-cadherin still interacts with EGFR was examined.

- To study the influence of mutant E-cadherin on EGFR internalization, FACS analyses were carried out. Examination of c-Cbl binding to EGFR after its activation supplemented this study.

- Beside the cell culture studies, activation of EGFR was determined in gastric carcinoma samples with E-cadherin mutations.

- E-cadherin mutations cause increased MMP-3 expression and secretion. The question whether the secreted MMP-3 zymogen is proteolytically active was clarified by zymography.

- Finally, the influence of mutant E-cadherin and MMP-3 on cell invasion was investigated.

2 Materials and Methods

Addresses of providers of the material used are listed in section 2.9.

2.1 Cell culture

2.1.1 Cell culture conditions

Cells were routinely cultured at 37 °C in a humidified 5% CO_2 atmosphere (Heraeus incubator type BBK 6220, Kendro Laboratory Products)

2.1.2 Cell lines and growth media

The following cell lines were used: MDA-MB-435S (human ductal carcinoma, American Type Culture Collection (ATCC) No. HTM-129™), A-431 (human epidermoid carcinoma, ATCC No. CRL-1555™), L-929 (mouse fibroblasts, ATCC No. CCL-1™). Two different clones were investigated for each E-cadherin expressing construct in this study.

The MDA-MB-435S strain evolved from the parent line MDA-MB-435, isolated from the pleural effusion of a female with metastatic, ductal adenocarcinoma of the breast (Cailleau et al., 1978). Recent advances in gene expression analysis revealed that the pattern for MDA-MB-435 more closely resembled that of melanoma cell lines than of other breast tumour lines (Ross et al., 2000). In addition, single nucleotide polymorphism (SNP) array analysis showed that MDA-MB-435 is derived from the same individual as the melanoma cell line M14 (Garraway et al., 2005). Until now it is a matter of debate whether MDA-MB-435 is a breast cancer or a melanoma cell line.

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, # 31966-021, Gibco), 10% fetal calf serum (FCS, # 3302-P260317, PAN Biotech) and 2.5 ml penicillin/ streptomycin (10,000 units/ml penicillin G sodium/10,000 µg/ml streptomycin sulphate; # 15140-122, Gibco). Transfected cells were grown in medium additionally supplemented with Geneticin G418 sulfate (600 mg/l, # 11811-031, Gibco). Tissue culture flasks and plates were purchased from Biochrom AG.

2.1.3 Passaging of cells

Cells were washed with prewarmed PBS (# H15-002, PAA Laboratories), trypsinized for approximately 5 min (0.5 ml Trypsin-EDTA (# L11-004, PAA Laboratories) per 25 cm^2 tissue culture flask, 37 °C), harvested in prewarmed culture medium (37 °C), splitted in an appropriate ratio and seeded in 25 cm^2 tissue culture flasks.

2.1.4 Preparation of frozen stocks

Cells grown to 90 % confluency in a 15 cm tissue culture plate were washed with PBS, trypsinized, harvested in culture medium (4 °C) and transferred to a 15 ml Falcon tube. After centrifugation at $300 \times g$ (4 °C) for 5 min, cells were resuspended in 2 ml DMEM supplemented with 20 % FCS. In each case, 500 µl of cell suspension were aliquoted into 2 ml cryo vials containing 500 µl DMEM supplemented with 20 % DMSO (# 20385, Serva). Cryo vials were placed at −20 °C for 4 h and subsequently transferred to −80 °C. The next day vials were transferred into liquid nitrogen for long time storage.

2.1.5 Starting culture from frozen stocks

25 cm^2 tissue culture flasks containing 5 ml culture medium (37 °C, without Geneticin G418) were prepared before thawing. Cryo vials with frozen cells were transferred to a 37 °C water bath. After thawing, cells were either seeded directly into the culture flasks or transferred to a medium containing tube, centrifuged at $300 \times g$ for 3 min, resuspended in fresh medium, and seeded in 25 cm^2 tissue culture flasks. The medium was changed 4 hours later. Geneticin G418 was added the next day.

2.1.6 Positive selection of E-cadherin expressing cells

To enrich MDA-MB-435S cells expressing E-cadherin variants, the *CELLection™ Pan Mouse IgG Kit* (# 115-31, Dynal Biotech, distributed by Invitrogen) was used. The desired volume of Dynabeads$^®$ was washed according to the manufacturer's instructions. Cells were detached with Versene (# 15040-033, Gibco) and 1×10^7 cells were resuspended in 1 ml Buffer 2. For pre-coating of washed Dynabeads$^®$, 0.5 µg anti-SHE-78-7 antibody (# TAK-M126, distributed by Axxora) was used per 25 µl Dynabeads$^®$. Cell isolation (direct technique) was carried out according to the manufacturer's instructions.

2.1.7 Detection of Mycoplasma in cultured cells

To detect mycoplasma in cultured cells, a nested PCR protocol (TaKaRa) was used. Three days after thawing cells, 100 µl of supernatant from each cell line (90 % confluent) were taken. The supernatant was heated to 96 °C for 5 min and debris was spun down (13,000 rpm for 5 sec). 10 µl of each sample was used as template for the first PCR and following components were added: 24.5 µl aqua dest, 5 µl 10 × Buffer (15 mM Mg^{2+}), 8 µl dNTPs (1.25 mM each; # 803-0007, Applied Biosystems), 2 µl Primer-Mix 1, and 0.5 µl Taq-Polymerase (5,000 U/ml; # 27079906, GE Healthcare). The samples were heated to 94 °C for 4 min. A cycle composed of DNA denaturation at 94 °C for 30 sec, primer annealing at 55 °C for 2 min and elongation at 72 °C for 1 min was repeated 35 times. Finally, the samples were heated to 72 °C for 7 min and subsequently stored at 4 °C. 1 µl of the first PCR was used as template for the second PCR and following components were added: 33.5 µl aqua dest, 5 µl 10 × Buffer (15 mM Mg^{2+}), 8 µl dNTPs (1.25 mM each), 2 µl Primer-Mix 2, and 0.5 µl Taq-Polymerase (5,000 U/ml). The samples were heated to 94 °C for 4 min. A cycle composed of DNA denaturation at 94 °C for 30 sec, primer annealing at 55 °C for 2 min and elongation at 72 °C for 1 min was repeated 30 times. Finally, the samples were heated to 72 °C for 7 min and 10 µl were loaded onto a 1.5 % agarose gel (# 840006, Biozym).

Table 2.1: Primer for Mycoplasma PCR (based on protocol from TaKaRa)

	Sequence 5' → 3'
Myco – F1	ACA CCA TGG GAG CTG GTA AT
Myco – F1t	ACA CCA TGG GAG TTG GTA AT
Myco – R1	CTT CAT CGA CTT TCA GAC CCA AGG CAT
Myco – R1tt	CTT CTT CGA CTT TCA GAC CCA AGG CAT
Myco – R1cat	CCT CAT CGA CTT TCA GAC CCA AGG CAT
Myco – R1ac	CTT CAT CGA CTT CCA GAC CCA AGG CAT
Myco – F2	GTT CTT TGA AAA CTG AAT
Myco – F2a	ATT CTT TGA AAA CTG AAT
Myco – F2cc	GCT CTT TCA AAA CTG AAT
Myco – R2	GCA TCC ACC AAA AAC TCT
Myco – R2ca	GCA TCC ACC ACA AAC TCT
Myco – R2at	GCA TCC ACC AAA TAC TCT

Table 2.2: Mycoplasma that can be detected (amplification size)

	1st PCR (bp)	2nd PCR (bp)
M. hyopneumoniae	681	237
M. neurolyticum	501	196
M. fermentans	491	195
M. hyorhinis	448	211
M. orale	423	179
M. arthritidis	408	157
M. salivarium	403	151
M. hominis	370, 369	147, 148
M. arginini	369	145

Primer-Mix 1: Myco-Primer F1 and F1t were mixed two-to-one with Myco-Primer R1, R1tt, R1cat and R1ac.

Primer-Mix 2:
Following Myco-Primer were mixed in equal shares: F2, F2a, F2cc, R2, R2ca, and R2at.

2.2 Western blot analysis

2.2.1 Cell lysis

Extraction of proteins from cultured cells at corresponding confluency was performed with L-CAM lysis buffer. 24 h after seeding, cells were washed twice with ice-cold PBS. Prechilled L-CAM lysis buffer was added (200 µl/10 cm tissue culture plate), adherent cells were scraped of and transferred into a 1.5 ml tube. Cell suspension was incubated on ice for 10 min and subsequently centrifuged at 13,000 rpm (4 °C) for 10 min. The supernatant was transferred to a fresh tube and stored at –80 °C. If cells were stimulated with growth factors, culture medium with 10 % FCS was removed 8 h after seeding and replaced by DMEM without FCS. After 16 h serum starvation, cells were treated with or without 100 ng/ml EGF (# 9644, Sigma), 50 ng/ml HB-EGF (# 100-47, PeproTech), or 20 ng/ml HGF (# 375228, Calbiochem) for the indicated period of time.

10 × L-CAM lysis buffer: 140 mM   NaCl (# 1.06404.5000, Merck)

47 mM   KCl (# 1.04936.0500, Merck)

7 mM $MgSO_4$ (# 80309, Fluka)

12 mM   CaCl2 (# 1016, Sigma)

10 mM   Hepes pH 7.4 (# 3375, Sigma)

Buffer was stored at room temperature.

1 × L-CAM lysis buffer: 10 % (v/v)   10 × L-CAM lysis buffer

1 % (v/v)   Triton X-100 (# 648462, Merck)

2 mM   PMSF (# 7626, Sigma)

2 mM Na_3VO_4 (# 6508, Sigma)

19 µg/ml   Aprotinin (# 6279, Sigma)

20 µg/ml   Leupeptin (# 2032, Sigma)

10 mM $Na_4P_2O_7 \times 10\ H_2O$ (# 221368, Sigma)

10 mM   NaF (# 7920, Sigma)

Immediately prior to cell lysis 1 × L-CAM buffer was prepared on ice.

2.2.2   Calcium-depletion procedure

Cell-cell contacts were disrupted by adding EGTA (# 4378, Sigma) to cells in culture to a final concentration of 2 mmol/L. Cells were incubated for 6 min at 37 °C and subsequently treated with or without growth factors. Protein extraction was performed as described above.

2.2.3   Protein quantification (based on the method of Bradford)

The protein quantification assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. *Protein Assay Dye Reagent Concentrate* (# 500-0006, Bio-Rad) was diluted 1:5 in aqua dest. Protein Standard II (1.48 mg BSA/ml, # 500-0007, Bio-Rad) was diluted 1:10 in aqua dest. Samples for a standard curve were prepared as shown in Table 2.3 and transferred into half-microcuvettes. In each case 995 µl of diluted *Protein Assay Dye Reagent Concentrate* were aliquoted in half-microcuvettes and 5 µl of test samples were added (dilution 1:200). After vortexing, all samples were incubated at room temperature for 3 min. The absorbance was measured at 595 nm vs. diluted *Protein Assay Dye Reagent Concentrate* reference, a standard curve was established, and the sample protein concentration was calculated.

Table 2.3: Sample preparation for standard curve

Sample #	Protein Assay Dye Reagent Concentrate (1:5 dilution in aqua dest.)	Protein Standard II [1.48mg BSA/ml] (1:10 dilution in aqua dest.)	Protein [µg]
1	990 µl	10 µl	1.48
2	970 µl	30 µl	4.44
3	950 µl	50 µl	7.4
4	930 µl	70 µl	10.36

2.2.4   SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

15-20 µg of proteins from each indicated extract were heated in $5 \times$ SDS-PAGE (Laemmli) sample buffer to 96 °C for 5 min and loaded onto an appropriate polyacrylamide minigel to separate proteins according to their molecular weight (*Mini-PROTEAN*® *3 Cell*, # 165-3301, Bio-Rad). Routinely, 3 µl *Dual Color Precision Plus Protein™ Standards* were loaded (# 161-0374, Bio-Rad).

Table 2.4: Gel formulations for SDS-PAGE

	Stacking gel (5ml)	Separation gel (10 %) (15 ml)
Aqua dest.	3.05 ml	7.33 ml
0.5 M Tris (pH 6.8)	---	3.75 ml
1 M Tris (pH 8.8)	1.25 ml	---
40 % Acrylamid/Bis (# 161-0148, Bio-Rad)	0.63 ml	3.75 ml
20 % SDS (# A0675.1000, AppliChem)	25 µl	75 µl
10 % APS (# A7460, Sigma-Aldrich)	40 µl	90 µl
TEMED (# 161-0801, Bio-Rad)	4 µl	9 µl

2.2.5 Immunoblotting

Proteins were transferred to either nitrocellulose membranes (# 10401396, Schleicher & Schuell) or PVDF membranes (# RPN303F, GE Healthcare) by using the *Mini Trans-Blot Cell* system (# 170-3935, Bio-Rad). PVDF-membranes were pre-incubated in methanol for 1 min and equilibrated in blot-buffer for 1 min at room temperature. Fiber pads and gel blotting papers (# 10426693, Schleicher & Schuell) were soaked in transfer buffer. The blot sandwich was prepared as follows: a fiber pad was laid on the black part of the blotting cassette (cathode). The SDS gel was carefully detached from the glass plate of the gel electrophoresis unit by a dry gel blotting paper, put on the fiber pad and covered with a membrane. The membrane was in turn covered with another gel blotting paper and a fiber pad. Together with a cooling unit, the cassette was placed in the buffer tank, completely covered with ice-cold transfer buffer. The transfer was carried out with a constant voltage of 100 V for 60 min. Following the transfer, the membrane was subjected to the blocking procedure.

2.2.6 Immunological detection of immobilized proteins

Non-specific sites on the membrane were blocked with blocking buffer for 1 h at 4°C. The membrane was incubated with primary antibody diluted in blocking buffer for 16 h (overnight) at 4°C with agitation. Following the blot was rinsed 3×10 min with TBST at room temperature. Incubation with horseradish peroxidase (HRP)-conjugated secondary antibody was performed for 2 h at 4°C with agitation. Subsequently the membrane was washed again 3×10 min in TBST. Detection of HRP activity was performed using either the *ECL-Plus Western Blotting Detection System* (# RPN2132, GE Healthcare) according to the manufacturer's instructions or a self-made buffer system. A film (# RPN 3103K, Amersham Biosciences) was exposed to the membrane in a film cassette for an appropriate period of time, developed and fixed in red light.

2.2.7 Stripping

Blots to be stripped were rinsed with TBST, placed in *Restore™ Western Blot Stripping Buffer* (# 21059, Pierce) and incubated for 10 min at room temperature. Thereafter the blots were washed again 3×10 min in TBST and subjected to immunodetection. Membranes that were not stripped immediately after chemiluminescent detection were stored in PBS at 4 °C until the stripping procedure was performed.

2.2.8 Antibodies

Table 2.5: Antibodies for Western blot analysis

Antibody	Dilution	Blocking buffer
Mouse anti-**c-Cbl** (# 610441, BD Biosciences)	1:1000	5 % non-fat dried milk
Mouse anti-**E-cadherin** (# 610181, BD Biosciences)	1:5000	5 % non-fat dried milk
Rabbit anti-**EGFR** pAb (1005, # sc-03, Santa Cruz)	1:1000 in TBST	5 % non-fat dried milk
Rabbit anti-**phospho-EGFR** [pY1068] pAb (# 44-788G, Biosource)	1:2000 in 3 % BSA	2.5 % non-fat dried milk / 0.5 % BSA
Rabbit anti-**phospho-EGFR** [pY1148] pAb (# 44-792G, Biosource)	1:2000 in 1 % BSA	2.5 % non-fat dried milk, 0.5 % BSA
Rabbit anti-**phospho-EGFR** [pY1173] pAb (# 44-794G, Biosource)	1:2000 in 3 % BSA	2.5 % non-fat dried milk, 0.5 % BSA
Rabbit anti-**phospho-EGFr** (Tyr1086) pAb (# 36-9700, Zymed)	1:2000	5 % BSA
Mouse anti-**GRB2** (# 610111, BD Biosciences)	1:1000	5 % non-fat dried milk
Mouse anti-**Met** (25H2) mAb (# 3127, Cell Signaling)	1:200	5 % non-fat dried milk
PhosphoDetect™ Anti-**c-Met** (pTyr1230/1234/1235) Rabbit pAB (# PS100, Calbiochem)	1:1000	5 % non-fat dried milk
Mouse anti-**MMP-3** (Ab-5) mAb (# IM70, Oncogene)	1:100	5 % non-fat dried milk
Mouse anti-**Ras** mAB (part of *EZ-Detect™ Ras Activation Kit*, # 89855, Pierce)	1:200	3 % BSA
Mouse anti-**SHC** (# 610878, BD Biosciences)	1:1000	5 % non-fat dried milk
Rabbit anti-**Src** pAb (# 2108, Cell Signaling)	1:1000 in 5 % BSA	5 % non-fat dried milk
Phospho-Src Family (Tyr416) pAb (# 2101, Cell Signaling)	1:1000 in 5 % BSA	5 % non-fat dried milk
Mouse anti-α-**Tubulin** mAb (# 9026, Sigma)	1:10,000	5 % non-fat dried milk
Goat F(ab')$_2$ Anti Rabbit Ig's – HRP (# ALI4404, Biosource)	1:40,000	according to the primary antibody
Sheep anti-mouse IgG – HRP (# NA931-1ML, GE Healthcare)	1:10,000	according to the primary antibody

2.2.9   Buffers and solutions for Western blot analysis

10 % APS:
1 g ammonium persulfate (# 7460, Sigma) was dissolved in 10 ml aqua dest; aliquots were stored at −20 °C.

5 × Laemmli buffer:	60 mM	1.25 M Tris-HCl (pH 6.8)
	8.7 % (v/v)	Glycerol (# 1.04092.1000, Merck)
	720 mM	2-Mercaptoethanol (# 7154, Sigma)
	2 % (w/v)	SDS (# 151213, USB)
	80 µg/ml	Bromphenol blue (# 5525, Sigma)

Dissolved with agitation in a 15 ml Falkon tube for 2 h at room temperature; stored in aliquots at 4 °C.

10 × SDS running buffer:	0.25 M	Tris base (# 75825, UBS)
	1.92 M	Glycine (# 1.04201.1000, Merck)
	1 % (v/v)	20 % SDS solution (# A0675.1000, AppliChem)

The buffer was stored at room temperature.

10 × Transfer buffer:	0.25 M	Tris base
	1.92 M	Glycine
	1 % (v/v)	20 % SDS solution (# A0675.1000, AppliChem)

The buffer was stored at room temperature. Use: 100 ml of 10 × transfer buffer were diluted with 700 ml aqua dest and 200 ml methanol (# 1.06009.1000, Merck) and stored at 4 °C.

10 × TBS (pH 7.3):	0.5 M	Tris base
	1.5 M	NaCl (# 1.06404.5000, Merck)

The pH was adjusted, the buffer was autoclaved and stored at room temperature.

TBST
TBS supplemented with 0.1% v/v Tween-20 (# 8.22184.1000, Merck). The buffer was stored at room temperature.

Blocking buffer:
TBST supplemented with the indicated percentage of non-fat dried milk (# 60219, Töpfer) or BSA (# 3912, Sigma). The buffer was prepared freshly before use and stored at 4 °C for.

Solution 1:	2.5 mM	Luminol (# 8511, Sigma)
(for HRP-activity detection)	400 µM	p-Coumaric acid (# 9008, Sigma)
	100 mM	Tris-HCl pH 8.6

Solution 2: 0.15 % (v/v) H$_2$O$_2$ (# 1.07209.0250, Merck)
(for HRP-activity detection) 100 mM Tris-HCl pH 8.6
Solutions 1 and 2 were stored at 4 °C.

2.3 Immunoprecipitation

Cells were seeded at a density of 2.5×10^6 on 10 cm tissue culture plates. DMEM supplemented with 10 % FCS was removed 8 h after seeding and replaced by DMEM without FCS. After 16 h serum starvation, cells were treated with or without EGF (100 ng/ml) for the indicated period of time prior to cell lysis. As a control, EGFR activation was blocked by application of 6.3 µmol/L Tyrphostin AG 1487 (# 4182, Sigma) which was added during the 16 h incubation in DMEM without FCS. 500 µg of protein lysate and 2 µg polyclonal anti-EGFR (1005: sc-03) antibody (Santa Cruz) were used for precipitation of EGFR with the *Catch and Release$^®$ v2.0 Reversible Immunoprecipitation System* (# 17-500, Upstate). Compared to traditional IP protocols which use Protein A or Protein G coupled to agarose beads, *Catch and Release$^®$* spin columns contain a resin slurry that binds the so-called antibody capture affinity ligand (ACAL). The ACAL in turn binds the antigen-antibody complex and serves as a tether between the complex and the resin resulting in complex immobilization in the column. After removing unbound non-specific proteins, bound immunocomplexes were eluted and subsequently subjected to immunoblotting analysis.

2.4 GST-Raf1-RBD pulldown assay

Cells were seeded at a density of 3×10^6 on 10 cm or 15 cm tissue culture plates. After 8 h, DMEM with 10 % FCS was replaced by DMEM without FCS. After 16 h serum starvation, cells were treated with or without 100 ng/ml EGF (# 9644, Sigma) for the indicated period of time before they were lysed. Using equal amounts of protein, the *EZ-Detect™ Ras Activation Kit* (# 89855, Pierce) was performed. Active Ras binds specifically to the Ras-binding domain (RBD) of Raf1, which can be used as a probe to specifically isolate the active form of Ras. The *EZ-Detect™ Ras Activation Kit* uses a GST-fusion protein containing RBD of Raf1 which was incubated with 750 µg of protein lysate in the presence of a *SwellGel$^®$* Immobilized Glutathione Disc. Unbound protein was removed an active Ras was subsequently pulled-down and detected by Western blot analysis using Anti-Ras antibody.

2.5 Quantitative indirect immunofluorescence assay

To quantify cell-surface E-cadherin by flow cytometry, an indirect immunofluorescence assay (QIFIKIT®, # K 0078, Dako) was performed. MDA-MB-435S and L929 transfectants were harvested with versene, and 5×10^5 cells were incubated with 40 µg/ml monoclonal antibody directed to E-cadherin (SHE78-7, Alexis) for 1 h on ice. Then, cells and calibration beads that were coated with different quantities of mouse monoclonal antibody molecules were labelled in parallel with FITC-conjugated goat anti-mouse IgG (diluted 1:50; delivered with the QIFIKIT) for 1 h on ice. Cells and calibration beads were analysed on a Beckman Coulter Epics XL. The antigen quantity was expressed in antibody binding capacity (ABC) units. The calibration beads were used for construction of the calibration curve against ABC. ABC of analysed cells was calculated based on the equation of the calibration curve.

2.6 Flow cytometry

To assess the surface localization of EGFR, cells were harvested with Versene (# 15040-033, Gibco) and 5×10^5 cells were incubated with 20 µg/ml monoclonal antibody directed to EGFR (BD Biosciences) for 1 h on ice in PBS, washed with PBS supplemented with 0.1 % sodium azide (# 106688, Merck) and 0.1 % bovine serum albumine (# 3912, Sigma) and stained with DTAF-conjugated anti mouse IgG for 1 h on ice. To examine the internalization of EGFR in response to EGF, cells were seeded at a density of 1 x 10^6 cells per 10 cm dish, serum starved overnight and treated with 100 ng/ml EGF for 4 h. Cells were harvested with Versene and 5 x 10^5 cells were incubated with 5 ng/ml monoclonal antibody directed to EGFR (Upstate) for 1 h on ice in PBS, washed with PBS supplemented with 0.1 % sodium azide and 0.1 % bovine serum albumine and stained with DTAF-conjugated anti mouse IgG secondary antibody for 1 h on ice. Purified mouse IgG1 (Pharmingen, distributed by BD Biosciences) were used as κ immunoglobulin isotype controls. Cells were analyzed on a Beckman Coulter Epics XL.

2.7 Immunohistochemistry

The following antibodies were used for immunohistochemistry: anti-phosphorylated EGFR (rabbit polyclonal antibody reacting with EGFR only when phosphorylated at tyrosine residue 1086, #36-9700, obtained from Zymed Laboratories and mouse monoclonal antibody reacting with EGFR only when phosphorylated at tyrosine residue 1068 (1H12), # 2236, Cell Signaling Technology, obtained from New England Biolabs, Frankfurt), monoclonal rat E-cad delta 8-1 detecting mutant E-cadherin protein lacking exon 8 (*del 8* mutation; Becker et al., 2002), monoclonal rat E-cad delta 9-1 detecting mutant E-cadherin protein lacking exon 9 (*del 9* mutation), clone 7E6 (Becker et al., 1999) and anti-E-cadherin antibody HECD-1 (Alexis).

Manual staining protocols were used for phosphorylated EGFR and E-cadherin staining with the following antibody dilutions: phosphorylated EGFR (Y1086) 1:50, phosphorylated EGFR (Y1068) 1:400, *del 8* E-cadherin 1:5, *del 9* E-cadherin undiluted and normal E-cadherin 1:500. Antigen retrieval was performed using citrate buffer pH 6 using a microwave (for pEGFR, 2 x 10 min) or a pressure cooker (for HECD-1 for 7 min). A peroxidase block (3 % H_2O_2 for 15 min at room temperature), an avidin biotin block (Vectastain, two times 15 min at room temperature) and blocking with 5 % anti-goat serum in Dako dilution solution (1 h, room temperature) were performed. Staining was carried out with LSAB-DAB or Fast red from Dako Diagnostika GmbH.

Immunohistochemical analysis of 10 pre-selected formalin-fixed, paraffin-embedded tumour specimen with the somatic *del 8* or *del 9* E-cadherin mutations was performed using antibodies E-cad delta 8-1 and E-cad delta 9-1. The mutation-specific E-cad delta 8-1 and delta 9-1 antibodies stained exclusively tumour cells. As positive controls, formalin-fixed and paraffin-embedded cell pellets from MDA-MB-435S cells transfected with *del 8* or *del 9* E-cadherin were used (Handschuh et al., 1999). pEGFR was detected applying anti-EGFR phosphospecific antibodies directed against tyrosine 1068 or 1086, respectively. The pEGFR staining was cytoplasmic and membranous. As positive controls, formalin-fixed and paraffin embedded cell pellets from EGF-treated A431 cells were used. Immunohistochemical staining of 10 diffuse and 10 intestinal gastric cancers without *del 8* or *del 9* E-cadherin reactivity was performed using a gastric cancer tissue microarray. pEGFR was detected as described above and E-cadherin was stained with the HECD-1 antibody. Tumours were considered as E-cadherin positive when they showed strong membranous staining. The protocol was

reviewed and approved by the local ethics committee and informed consent was obtained according to institutional regulations.

2.8 Invasion assay

To study cell invasion of MDA-MB-435S transfectants, *BioCoat™ Matrigel™ Invasion Chambers* (# 354480, BD Biosciences) were used. The invasion chambers consist of *Falcon Cell Culture Inserts* which contain an 8 µm pore size membrane with a layer of *Matrigel* matrix. Only invasive cells are able to detach themselves from and migrate through this matrix. In advance, the required number of inserts were removed from −20 °C storage and allowed to come to room temperature. Pre-warmed DMEM was added to the interior of the inserts and bottom of wells (*Falcon TC Companion Plate*) and allowed to rehydrate for 2 h at 37 °C in a humidified 5% CO_2 atmosphere. Subsequently, chemoattractant (DMEM supplemented with 20 % FCS) was transferred to the wells of the *Falcon TC Companion Plate*. 2.5×10^4 cells (in 500 µl DMEM with 10 % FCS) were added to each insert and incubated for 22 h at 37 °C in a humidified 5% CO_2 atmosphere. After removing non-invading cells at the top side of the membranes, membranes were detached from the insert housing. Cell invasion was measured by staining and counting the remaining invasive cells at the bottom side of the membranes.

2.9 Zymography

Cells were seeded at a density of 2×10^5 in 12-well tissue culture plates. DMEM supplemented with 10 % FCS was removed 6 h after seeding and replaced by 750 µl per well DMEM without FCS. 72 h after seeding supernatants were transferred into 1.5 ml tubes and centrifuged at 13,000 rpm (4 °C) for 5 min to remove debris and dead cells. In each case 250 µl supernatant were incubated with 1 ml ice-cold acetone for 30 min on ice to precipitate proteins. After centrifugation at 14,000 rpm (4 °C) for 30 min, supernatants were discarded and pellets were resuspended in 40 µl zymography loading buffer without boiling. 30 µl of each sample were loaded onto a 0.05 % Casein/10 % polyacrylamide minigel to separate proteins according to their molecular weight (Table 2.6). Following electrophoresis, the zymogram was rinsed twice with washing buffer and once with aqua dest to remove the SDS. The zymogram was incubated in substrate buffer at 37 °C overnight. Subsequently, the zymogram was stained with Coomassie Brilliant Blue G-250 (# 17525, Serva) and destained to detect

the areas of digestion which appeared as clear bands against the darkly stained background where the substrate has been degraded by the enzyme.

Table 2.6: 0.05 % Casein / 10 % PA gel for zymography

	Stacking gel	Separation gel 0.05 % Casein / 10 % PA
Aqua dest.	3.05 ml	7.33 ml
1 % Casein (#1.02241.1000, Merck)	---	0.5 ml
0.5 M Tris (pH 6.8)	---	3.75 ml
1 M Tris (pH 8.8)	1.25 ml	---
40 % Acrylamide/Bis (# 161-0148, Bio-Rad)	0.63 ml	3.75 ml
20 % SDS (# A0675.1000, AppliChem)	25 µl	75 µl
10 % APS (# A7460, Sigma-Aldrich)	40 µl	90 µl
TEMED (# 161-0801, Bio-Rad)	4 µl	9 µl

2.9.1 Buffers for zymography

1 % Casein
0.1 g Casein soluble in alkali (# 1.02241.1000, Merck) was dissolved in 10 ml 1.5 M Tris (pH 8.8) with agitation for 5 h at room temperature. The solution was stored at 4 °C.

Zymography sample buffer: 5 g   SDS (# 151213, USB)
                                 2 g   Sucrose (# 84100, Fluka)
                            0.25 M   Tris (pH 6.8)
Aliquots were stored at −20 °C.

Washing buffer:
2 % (v/v) Triton X-100 (# 648462, Merck) were diluted in aqua dest. The buffer was stored at room temperature.

MMP substrate buffer: 50 mM   Tris base (# 75825, UBS Corporation)
                             10 mM $CaCl_2 \times 2\,H_2O$ (# 7902, Sigma)
The buffer was stored at 4 °C.

Staining solution: 0.25 % (w/v)   Coomassie Brilliant Blue R250 (# 17525, Serva)
                           45 % (v/v)   Methanol (# 1.06009.1000, Merck)
                            9 % (v/v)   Acetic acid (# 1.00066.1000, Merck)

Coomassie powder was dissolved in methanol with agitation for 4 h. Subsequently, aqua dest was added and the buffer was stirred another 30-60 min. Buffer was stored in a dark bottle at room temperature.

Destaining solution 1:   45 % (v/v)   Methanol
                            7.5 % (v/v)   Acetic acid

Destaining solution 2:   10 % (v/v)   Methanol
                            7.5 % (v/v)   Acetic acid

Destaining solution 1 and 2 were stored at room temperature.

2.10 List of providers

Alexis Deutschland, Grünberg, Germany

Amersham Biosciences, Buckinghamshire, UK

AppliChem, Darmstadt, Germany

Applied Biosystems, Foster City, CA, USA

Axxora Deutschland GmbH, Lörrach, Germany

BD Biosciences Pharmigen, San Diego, CA, USA

Beckman Coulter GmbH, Krefeld, Germany

Biochrom AG, Berlin, Germany

BioRad Laboratories, Hercules, CA, USA

Biozym Scientific GmbH, Hess. Oldendorf, Germany

Dynal Biotech ASA, Oslo, Norway

Calbiochem, San Diego, CA, USA

Cell Signaling Technologies, Danvers, MA, USA

DakoCytomation, Glostrup, Denmark

GE Healthcare, Buckinghamshire, UK

Gibco, Paisley, Scotland/UK

Invitrogen, Karlsruhe, Germany

Kendro Laboratory Products GmbH, Langenselbold, Germany

Merck KGaA, Darmstadt, Germany

PAA Laboratories, Pasching, Austria

PAN Biotech, Aidenbach, Germany

PeproTech EC Ltd., London, UK

PIERCE, Rockford, IL, USA

Roche Diagnostics, Mannheim, Germany

Schleicher&Schuell Bioscience, Dassel, Germany

SERVA Electrophoresis GmbH, Heidelberg, Germany

Sigma-Aldrich, Munich, Germany

Tocris Bioscience, Bristol, UK

Töpfer GmbH, Dietmannsried, Germany

Upstate, Charlottesville, VA, USA

USB Corporation, Cleveland, OH, USA

Vector Laboratories, Burlingame, CA, USA

Zeiss, Jena, Germany

3 Results

3.1 Receptor crosstalk between EGFR and mutant E-cadherin

3.1.1 Effect of E-cadherin mutations on EGFR expression

In the present study, an established cell system was used to analyse the *crosstalk* between mutant E-cadherin and EGFR. *Wild-type (wt)* and mutant E-cadherin cDNAs were isolated previously from non-tumourous gastric mucosa or somatic gastric carcinoma of the diffuse type and stably expressed in MDA-MB-435S carcinoma cells (Handschuh et al., 1999). The effect of E-cadherin variants on EGFR protein expression was determined (Figure 3.1). EGFR levels increased in the presence of *wt* or mutant E-cadherin compared to mock-transfected MDA-MB-435S cells, suggesting up-regulation or stabilization of EGFR. In general, the protein level of EGFR is relatively low in this cell system. As shown before by immunoblot analysis, the migration pattern of *del 8* and *del 9* (deletions of exon 8 or 9) E-cadherin differs from that of the 120 kDa *wt* and *p8* (point mutation D370A in exon 8) E-cadherin proteins (Handschuh et al., 1999). *del 8* E-cadherin shows lower molecular mass than *wt* E-cadherin and additional smaller fragments of around 80 kD are detectable which presumably correspond to degradation products. *del 9* E-cadherin migrates with an apparent higher molecular mass than the *wt* protein. The expression of α-tubulin was examined as a loading control.

A

B

Deletion of exon 8 (*del 8*) or exon 9 (*del 9*)

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |

Point mutation in exon 8 *(p8)*

DTN<u>D</u> → DTN<u>A</u>

TM

Mature protein

EC 1 – 5

Cyto
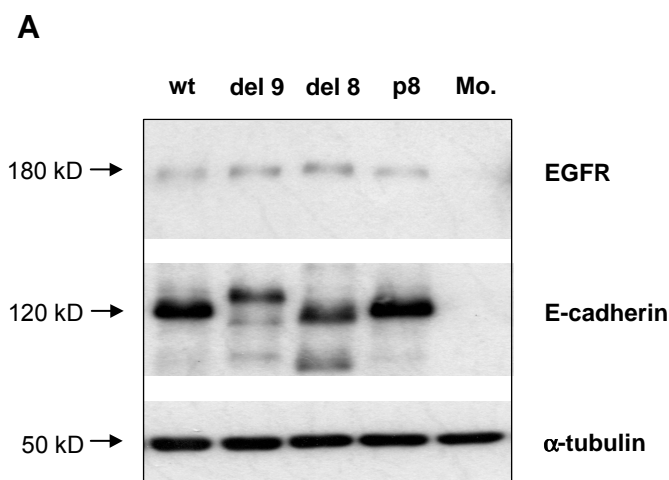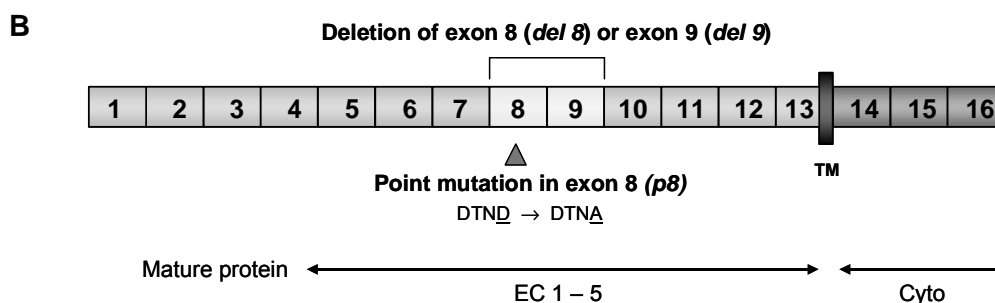
Figure 3.1: **Expression of E-cadherin and EGFR in MDA-MB-435S cells. (A)** E-cadherin and EGFR protein levels in *wt* or mutant (*del 9, del 8, p8*) E-cadherin expressing MDA-MB-435S cells were detected by Western blot analysis applying antibodies against E-cadherin and EGFR. Additionally, the blot was hybridised with anti-α-tubulin antibody to demonstrate equal loading in each lane. Mo.: mock transfected cells. **(B)** Exon structure of E-cadherin indicating gastric carcinoma derived mutations, which were analysed in this study .
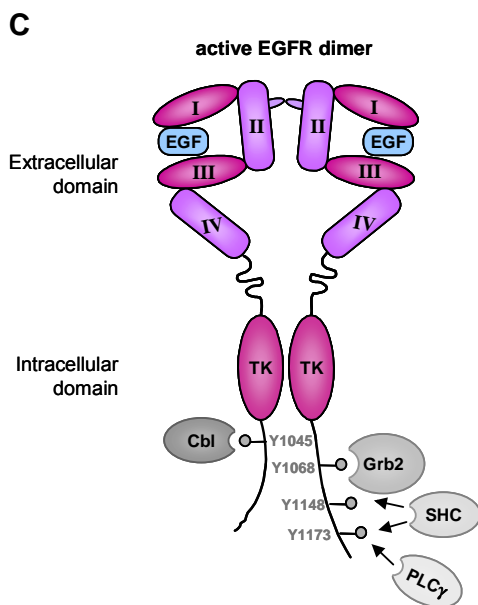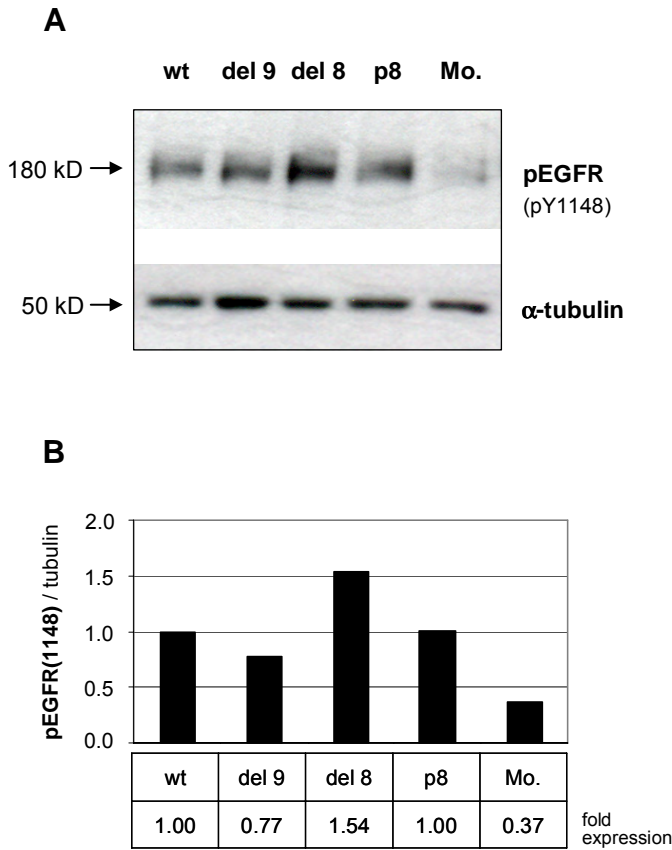
3.1.2   Enhanced activation of EGFR caused by somatic E-cadherin mutations

It has been reported recently that E-cadherin can negatively regulate ligand-dependent activation of several RTKs in an adhesion-dependent manner (Qian et al., 2004). Consequently the question arose whether tumour-derived somatic E-cadherin mutations interfere with this regulatory mechanism and influence EGF-dependent EGFR activation. Thus, the phosphorylation levels of EGFR in MDA-MB-435S cells expressing different E-cadherin variants were analysed by immunoblotting using phospho-epitope specific antibodies. Cells at 40 % confluency were treated with EGF for 1 min before they were examined. The strongest phosphorylation of EGFR at tyrosine (Y) 1148 was observed in the presence of *del 8* E-cadherin (Figure 3.2 A/B). Weaker EGFR activation was shown in *del 9* E-cadherin expressing cells as well as mock transfected cells compared to cells harbouring *wt* or *p8* E-cadherin. Because ligand binding and subsequent receptor dimerization leads to covalent attachment of a phosphate group to multiple tyrosine residues, further antibodies recognizing EGFR phosphorylated at Y1068 and Y1173 were used (Figure 3.2 C/D). Signalling and adapter molecules are recruited differently to phospho-tyrosines, thereby promoting diverse downstream signalling pathways. Interestingly, the phosphorylation intensities of the described tyrosine residues were not identical within one cell line. Cells expressing *del 8* or *p8* E-cadherin showed enhanced EGFR phosphorylation at Y1068 and Y1173 compared to *wt* E-cadherin expressing cells. In the presence of *del 9* E-cadherin EGFR activation was decreased. Taken together, these results suggest that

the activity level of EGFR is increased in the presence of somatic E-cadherin mutations affecting exon 8.

A

B

	wt	del 9	del 8	p8	Mo.	
fold expression	1.00	0.77	1.54	1.00	0.37	

C

active EGFR dimer

D

	wt	del 9	del 8	p8	Mo.	
	1.00	0.86	1.61	1.93	0.75	fold expression

E

	wt	del 9	del 8	p8	Mo.	
	1.00	0.80	1.39	1.43	0.59	fold expression
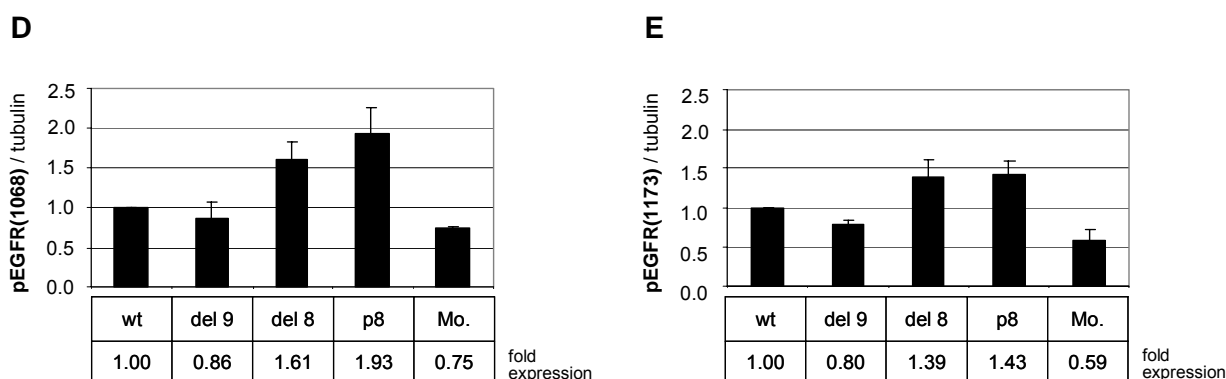
Figure 3.2: Enhanced phosphorylation of EGFR in the presence of somatic E-cadherin mutations. **(A)** EGFR activation was detected in *wt* and mutant (*del 9, del 8, p8*) E-cadherin expressing MDA-MB-435S cells. Transfectants were serum starved and stimulated with 100 ng/ml EGF for 1 min before they were subjected to immunoblot analysis using a phospho-epitope specific anti-EGFR (pY1148) antibody. **(B)** Western blot of (A) was quantified by densitometric analysis using Scion Image Software. **(C)** Different EGFR tyrosine phosphorylation sites and corresponding adapter molecules. The result obtained in (A) was validated with antibodies recognising EGFR phosphorylated at Y1068 **(D)** and Y1173 **(E)**. Each bar represents the mean $\pm$ SD of three independent experiments. Mo.: mock transfected cells

3.1.3 Kinetic of EGFR activation

Detailed kinetic analysis of EGF-dependent phosphorylation of EGFR was performed in *wt* and *del 8* E-cadherin expressing MDA-MB-435S cells at 40 % confluency. Cells were treated with EGF for different times within the range of 1-60 min to gain more information about the receptor activation characteristics. Transient phosphorylation of EGFR at tyrosine residue 1173 was observed with a maximum at 1 min stimulation time, followed by a rapid decrease (Figure 3.3 A/B). The course of EGFR activation was strongly influenced by the presence of the E-cadherin mutation because the level of phoshorylated EGFR at Y1173 was 47.3-fold elevated in *del 8* cells compared to 17.6-fold in *wt* cells. This result was confirmed with antibodies recognizing EGFR phosphorylated at tyrosine residues 1068 and 1148 (Figure 3.3 C). Notably, the expression levels of EGFR and E-cadherin remained unchanged during the time of stimulation (Figure 3.3 D). These findings imply that the rapid decrease in phosphorylation is not due to EGFR degradation or changes in E-cadherin protein levels.

A

B

0	1	2	3	5	60	0	1	2	3	5	60	EGF (min)
0.06	1.0	0.88	0.06	0.17	0.05	0.05	2.68	0.92	0.53	0.19	0.07	Fold expression

C

D
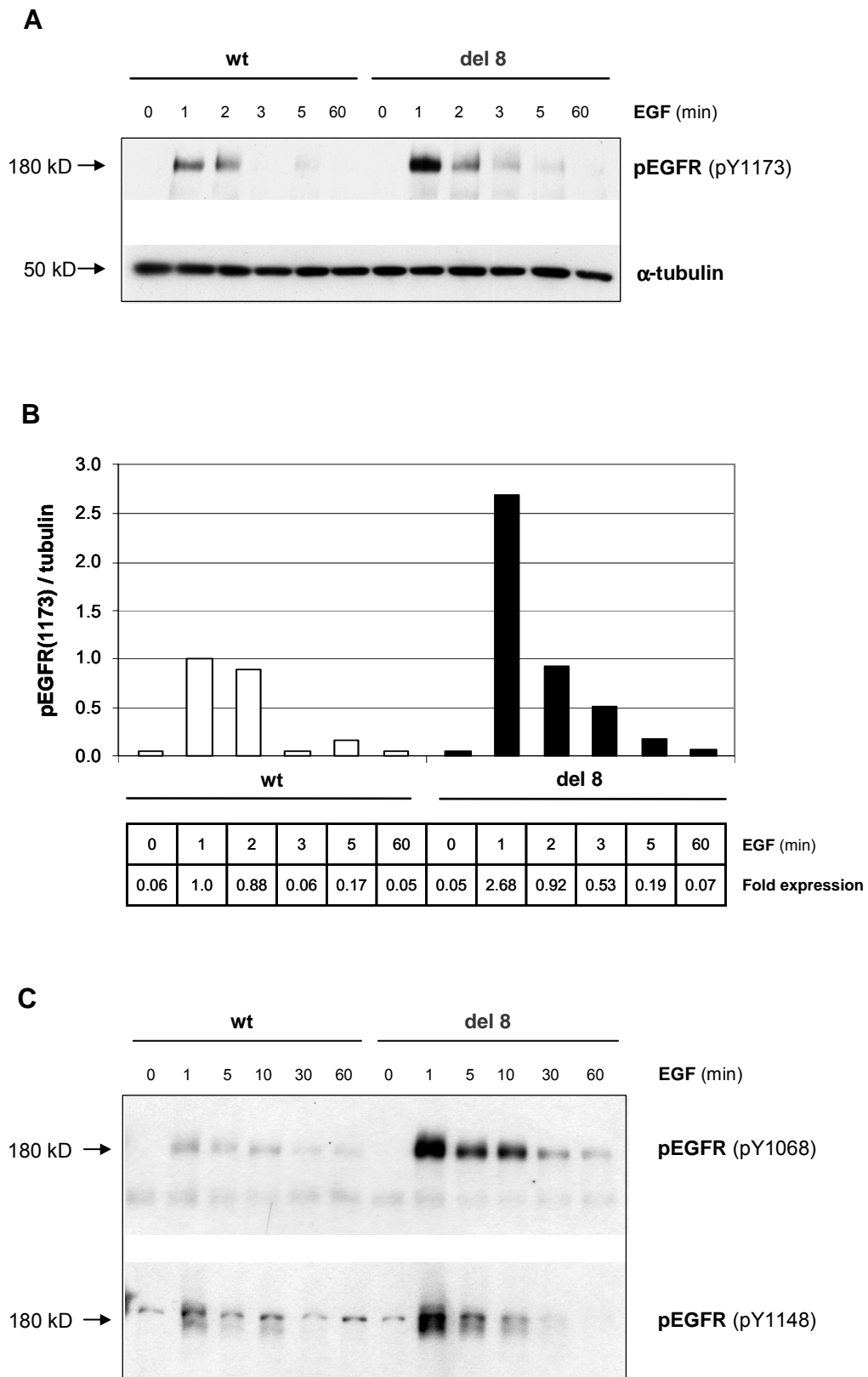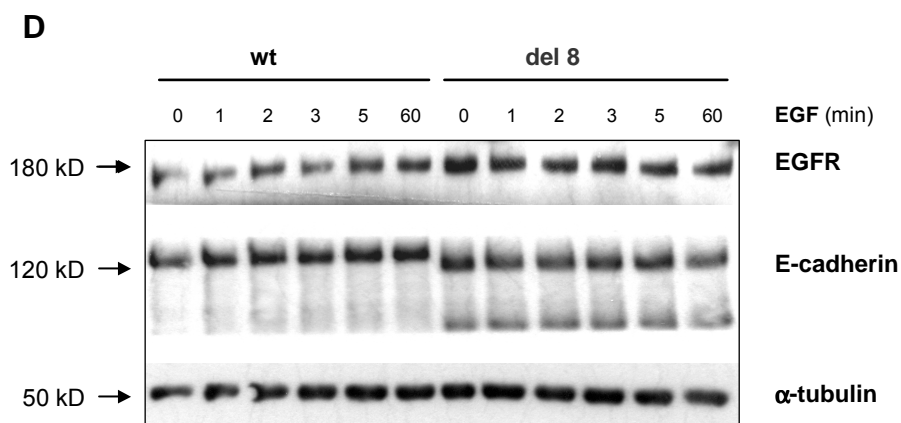
Figure 3.3: Kinetic analysis of EGF-dependent activation of EGFR. (A) Serum starved *wt* or *del 8* E-cadherin expressing MDA-MB-435S cells were stimulated with 100 ng/ml EGF for the indicated times and phosphorylation of EGFR was determined using an antibody recognising EGFR phosphorylated at tyrosine residue 1173. Blot was stripped and rehybridised with α-tubulin antibody to demonstrate equal loading in each lane. Shown is one representative of five independent experiments. **(B)** Immunoblot analysis of (A) was quantified by densitometric analysis using Scion Image Software. **(C)** The result obtained in (A) was confirmed with additional phospho-epitope specific antibodies. **(D)** Expression of EGFR and E-cadherin remained unchanged during EGF stimulation. **(E)** Morphology of MDA-MB-435S transfectants at 40 % cell confluency.

3.1.4 Activation of EGFR is sensitive to treatment with EGFR kinase inhibitor Tyrphostin AG 1478

To confirm the observed effects on EGFR phosphorylation in the presence of *wt* and mutant E-cadherin and to exclude unspecific binding of the utilised antibodies, the pharmacological EGFR inhibitor Tyrphostin AG 1478 was applied. MDA-MB-435S cells expressing *wt* or *del 8* E-cadherin were treated with the inhibitor and subsequently stimulated with EGF before they were subjected to immunoblotting analysis. Activation of EGFR was inhibited under these conditions, compared to EGF-stimulated cells without Tyrphostin AG 1478 treatment (Figure 3.4). Additionally, almost no EGFR phosphorylation was detected in untreated cells or in cells treated only with the inhibitor. In summary, these data verify the observed enhancement of EGFR activation in the presence of mutant E-cadherin and the specificity of phospho-epitope specific antibodies.

wt   del 8   wt   del 8   wt   del 8   wt   del 8

180 kD →

pEGFR (pY1173)

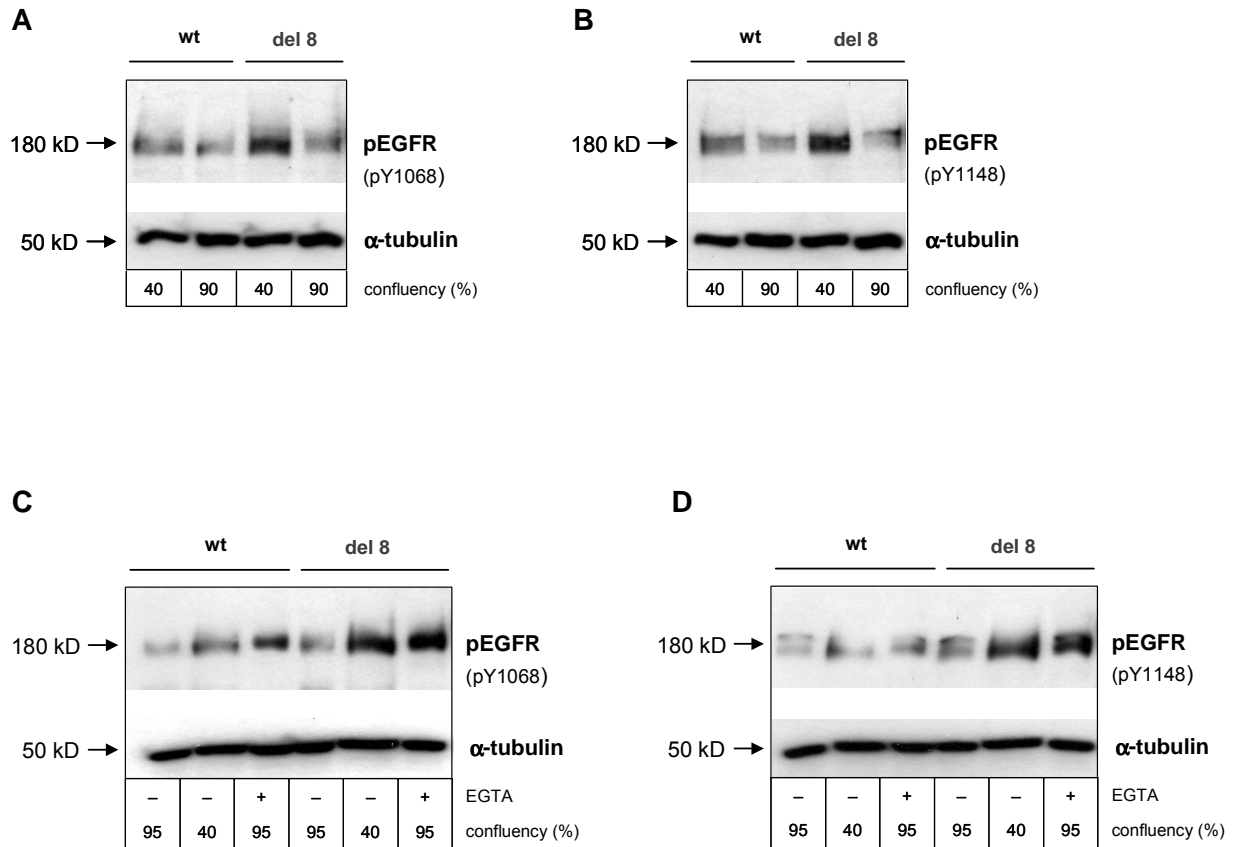| – | – | – | – | + | + | + | + | EGF |
| – | – | + | + | – | – | + | + | Tyrphostin |
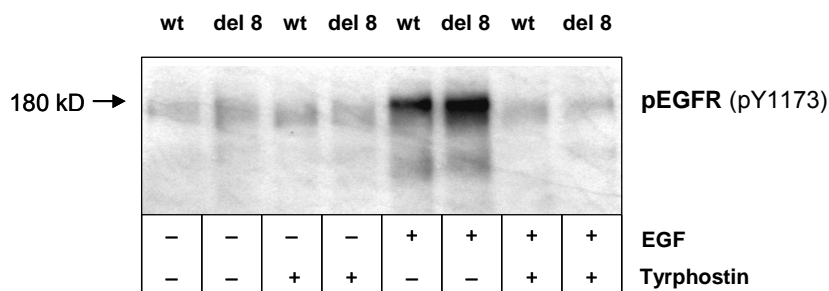
Figure 3.4: Tyrphostin AG 1478 inhibits EGFR phosphorylation. Influence of Tyrphostin AG 1478 on EGFR activation was examined by Western blot analysis in *wt* or *del 8* E-cadherin expressing cell. Serum starved transfectants were treated with Tyrphostin AG 1478 and subsequently stimulated with EGF. Untreated cells as well as EGF or Tyrphostin AG 1478 treated cells were used as controls.

3.1.5   EGF-induced EGFR activation is cell density dependent

Next, the influence of cell density and calcium depletion on EGF-induced phosphorylation of EGFR was studied to clarify the role of adhesive cell-cell contacts. MDA-MB-435S transfectants seeded at different cell confluencies were tested for these experiments. EGFR phosphorylation was dependent on cell density and was in general higher in low density (40 % confluency) than in high density (95 % confluency) *wt* and *del 8* cells (Figure 3.5 A/B). Again, EGF-dependent tyrosine phosphorylation of EGFR was stronger in *del 8* than in *wt* E-cadherin expressing cells. This effect was prominent in low density cells, but it was still detectable in confluent cells. To examine whether the lower response to EGF at high density was dependent on E-cadherin (*wt* or *del 8*) cell-cell adhesion, high-density cells were incubated with EGTA to deplete calcium. Reduction of extracellular calcium disrupts cell-cell contacts and induces the internalization of E-cadherin. Under these conditions, EGFR activation was increased as compared to untreated high-density cells and was almost as strong as in low density cells with little surface E-cadherin (Figure 3.5 D/E). The data obtained in this cell system confirm previous reports that *wt* E-cadherin can negatively regulate EGFR activation (Qian et al., 2004). Moreover, they demonstrate that the cell-density dependent EGFR-regulating function is preserved in mutant E-cadherin. The largest differences in EGFR activation between *wt* and mutant E-cadherin were observed under subconfluent conditions where *wt* cells form more E-cadherin-mediated cell-cell contacts than *del 8* cells (Handschuh et al., 1999). This result together with the calcium

depletion experiment indicates that the effect of E-cadherin on EGFR is adhesion-dependent.

A

| 40 | 90 | 40 | 90 | confluency (%) |

B

| 40 | 90 | 40 | 90 | confluency (%) |

C

| – | – | + | – | – | + | EGTA |
| 95 | 40 | 95 | 95 | 40 | 95 | confluency (%) |

D

| – | – | + | – | – | + | EGTA |
| 95 | 40 | 95 | 95 | 40 | 95 | confluency (%) |

Figure 3.5: Influence of confluency and calcium depletion on EGFR activation. (A/B) Serum starved *wt* or *del 8* E-cadherin expressing MDA-MB-435S cells were treated with 100 ng/ml EGF for 1 min and phosphorylation of EGFR at tyrosine residue 1068 or 1148 was analysed to determine the effect of different cell densities on EGFR activation. In each case one representative of three independent experiments is shown. **(C/D)** The experiment was performed as described in (A) and EGTA was included to disrupt E-cadherin-mediated cell-cell contacts in high-density cells. In each case one representative of two independent experiments is shown.

3.1.6   Activation of EGFR downstream signalling

Binding of EGF to the extracellular domain of EGFR causes dimerization of the receptor and its phosphorylation on several tyrosine residues within the cytoplasmic domain. The phosphorylated tyrosine residues serve as specific docking sites for the Src homology-2 (SH2) or phosphotyrosine binding domains of intracellular signal

transducers and adapters, leading to their colocalization and the assembly of multicomponent signalling complexes (Schlessinger, 2000). For further evaluation of the enhanced EGFR activation in the presence of mutant E-cadherin, the interaction of EGFR and the adapter proteins Grb2 and Shc which are recruited to the receptor after its activation was explored.

Cell extracts of untreated and EGF-stimulated *wt* and *del 8* E-cadherin expressing MDA-MB-435S cells were immunoprecipitated with anti-EGFR antibody and binding of Grb2 and Shc was examined by Western blot analysis. Consistent with the enhanced tyrosine phosphorylation of EGFR in *del 8* cells, the recruitment of both Grb2 and Shc to EGFR was stronger in *del 8* E-cadherin expressing cells than in *wt* E-cadherin transfectants (Figure 3.6 A). Interestingly, in unstimulated cells binding of both adapters to EGFR was detectable, suggesting that there is a basal level of activated EGFR in the cells. Beside EGFR, the adaptor molecules Grb2 and Shc are recruited to other activated receptors to transduce signals received at the membrane. In order to demonstrate specific involvement of EGFR in the analyzed downstream signalling, recruitment of Shc as an example was shown to be sensitive to treatment with the EGFR inhibitor Tyrphostin AG 1478 (Figure 3.6 C). These findings point out that EGFR activation is reflected by enhanced binding of Grb2 and Shc to the receptor.

A

IP: anti-EGFR
IB: anti-Shc, anti-Grb2

B

Input

C

IP: anti-EGFR
IB: anti-Shc

D

Input
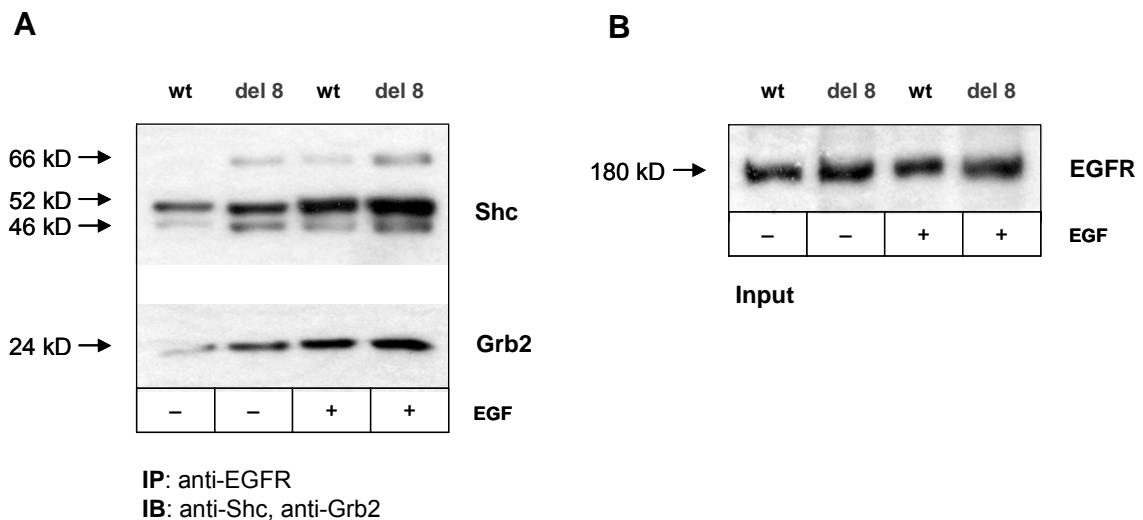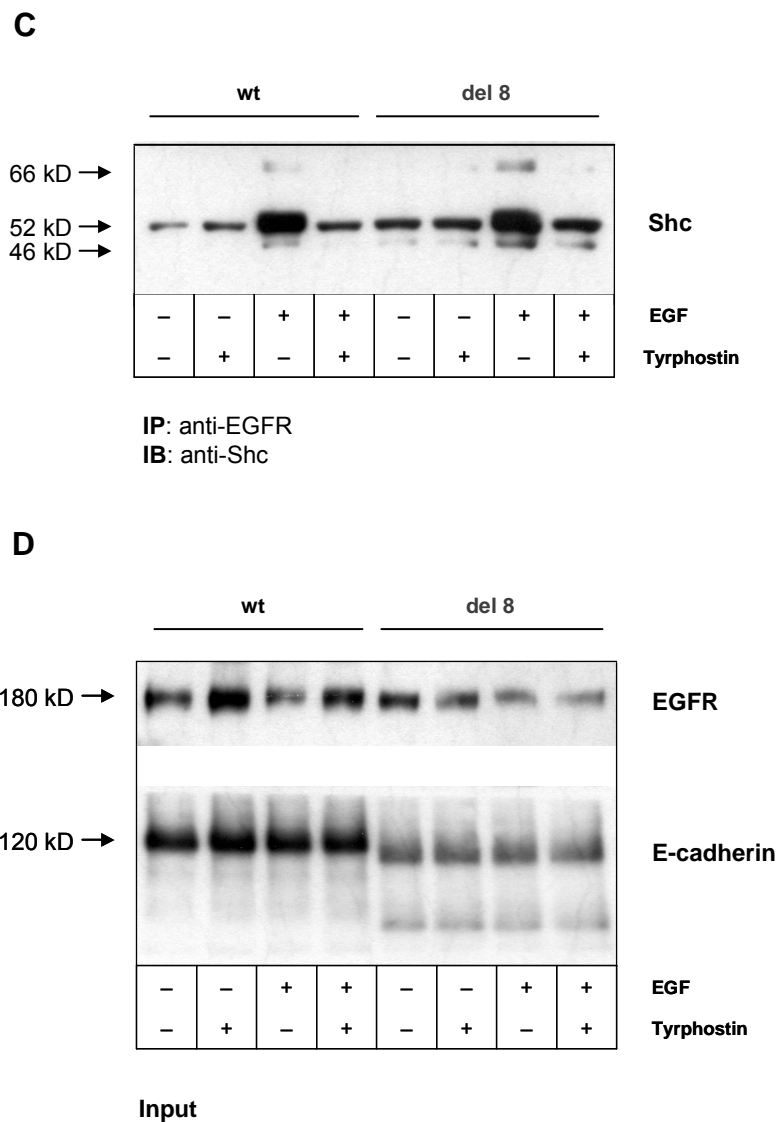
Figure 3.6: Recruitment of Shc and Grb2 after EGFR activation. (A) Binding of Shc and Grb2 to activated EGFR was investigated in *wt* or *del 8* E-cadherin expressing MDA-MB-435S cells. Extracts of serum starved unstimulated and EGF stimulated cells were immunoprecipitated with anti-EGFR antibody followed by immunoblotting with anti-Shc and anti-Grb2 antibodies or **(B)** directly analysed using anti-EGFR antibody as loading control (Input). Shown is one respresentative of three independent experiments. **(C)** The experiment was performed as described in (A) and Tyrphostin AG 1478 was included as a control. **(D)** Loading control.

3.1.7   Mutant E-cadherin increases the level of active Ras

Following the activation of the EGFR, Grb2, which is constitutively bound to the Ras exchange factor Sos, is recruited from the cytosol to the receptor at the membrane. This relocation of the Grb2/Sos complex facilitates the interaction of membrane-associated Ras with Sos, resulting in the exchange of Ras-bound GDP for GTP (Aronheim et al., 1994). Ras-GTP binds specifically to the Ras binding domain (RBD) of its effector molecule Raf1. Therefore, the RBD of Raf1 can be used as a probe to specifically isolate the active form of Ras. To further analyse the influence of mutant E-cadherin on downstream intracellular signalling after EGFR activation, the level of active Ras was determined in the presence of both *wt* and *del 8* E-cadherin.

Raf1-RBD pull-down assays were performed using untreated and EGF-stimulated MDA-MB-435S transfectants seeded at different cell densities (Figure 3.7). In the unstimulated situation, higher levels of active Ras were found in *del 8* than in *wt* E-cadherin expressing cells. After EGF-stimulation, the level of Ras-GTP in *wt* E-cadherin expressing cells increased to the level observed in *del 8* E-cadherin transfectants. In both cell lines, the Ras-GTP level was elevated in low-density cells compared to high-density cells. The differences at low versus high density were not related to changes in the expression levels of Ras or E-cadherin (Figure 3.7 and data not shown). These results suggest that mutant E-cadherin-dependent EGFR activation is mirrored by a higher level of active Ras.

B

Pull-down assay with GST-Raf1-RBD

	wt		del 8		wt		del 8		controls	
21 kD →										**Ras-GTP**
EGF	−	−	−	−	+	+	+	+		
conflueny (%)	85	40	85	40	85	40	85	40		

C

Input

	wt		del 8		wt		del 8		
21 kD →									**Ras**
	−	−	−	−	+	+	+	+	EGF
	85	40	85	40	85	40	85	40	confluency (%)
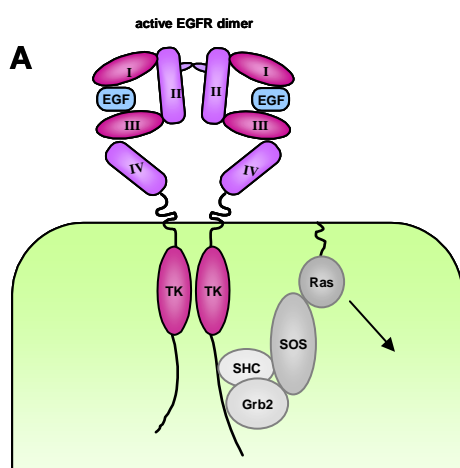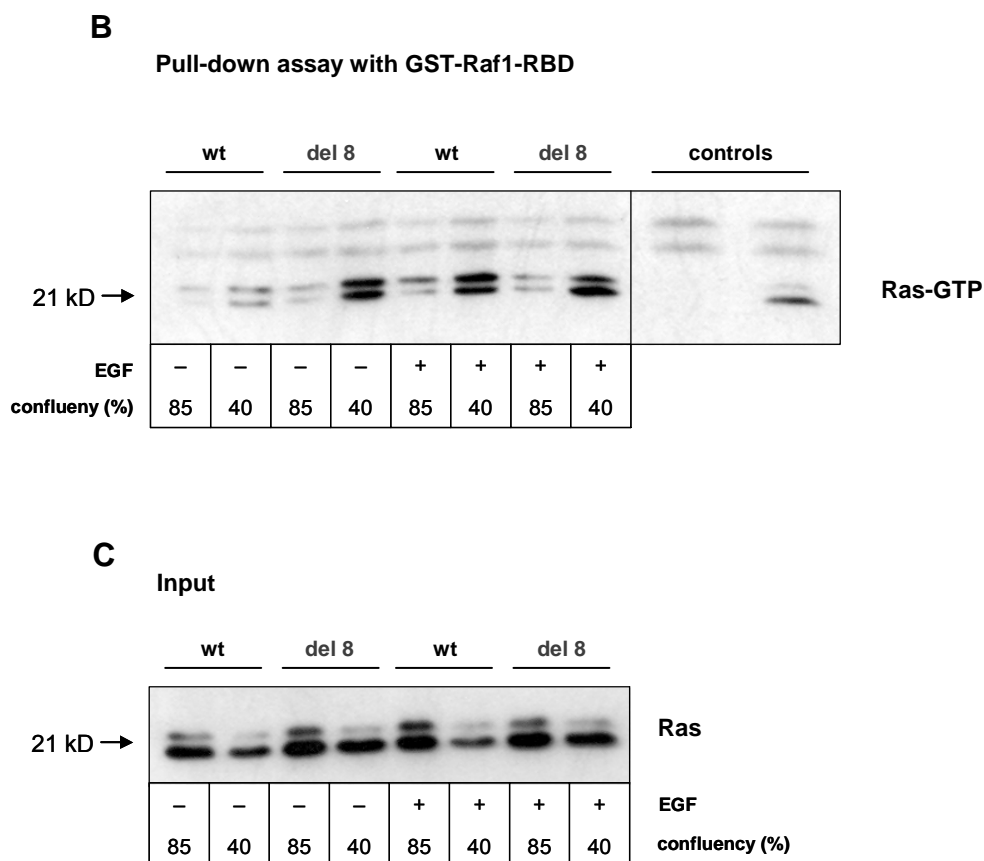
Figure 3.7: Activation of Ras. (A) The level of activated Ras influenced by different cell densities and EGF treatment was determined in *wt* or *del 8* E-cadherin expressing MDA-MB-435S cells by GST-Raf1-RBD pull-down assay. **(B)** The total amount of Ras was analysed by immunoblotting with anti-Ras antibody (Input). Shown is one representative of three independent experiments. **(C)** The interaction of membrane-associated Ras with SOS results in Ras activation by the exchange of Ras-bound GDP for GTP.

3.1.8 EGFR forms a complex with *wt* and *del 8* E-cadherin

E-cadherin was described to form a multicomponent complex with EGFR (Hoschuetzky et al., 1994). Several groups have demonstrated that the extracellular domain of E-cadherin is critical for this interaction (Fedor-Chaiken et al., 2003; Qian et al., 2004). In the light of the involvement of E-cadherin in regulation of EGFR activation, the physical interaction between both molecules was characterized. Complex formation of EGFR with either *wt* or *del 8* E-cadherin was observed by immunoprecipitation using anti-EGFR antibody under serum starvation conditions as well as during EGF treatment (Figure 3.8). This result indicates that deletion of exon 8 in the extracellular domain of

E-cadherin still permits interaction of mutant E-cadherin with EGFR. Of note, association of *del 8* E-cadherin with EGFR was weaker compared to the *wt* protein. Expression levels of EGFR and E-cadherin remained constant under these conditions (data not shown). Reduced binding of mutant E-cadherin to EGFR in a multicomponent complex or reduced stability of the complex may enhance EGFR surface motility, thereby facilitating EGFR dimerization and activation.

| wt		del 8		
				E-cadherin
−	+	−	+	EGF
1.0	0.8	0.7	0.5	fold binding

IP: anti-EGFR
IB: anti-E-cadherin
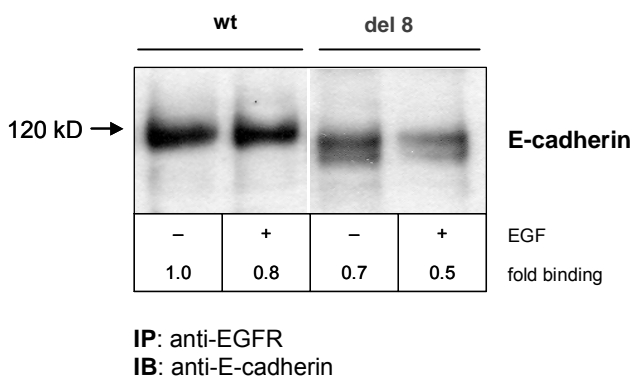
Figure 3.8: Complex formation between E-cadherin and EGFR. Association of *wt* and *del 8* E-cadherin with EGFR was investigated by immunoprecipitation of EGFR, followed by immunoblotting with an antibody directed to E-cadherin. Cells were cultured under serum starvation conditions or under EGF treatment for 1 min. Shown is one representative of four independent experiments.

3.1.9 Mutant E-cadherin shows decreased cell surface localization

Cell-cell contact-dependent inhibition of endocytosis contributes to the stabilization of E-cadherin at the cell surface (Le et al., 1999). Because the *del 8* mutation results in reduced cell-cell contact formation (Handschuh et al., 1999), decreased *del 8* E-cadherin surface levels were expected. To test this hypothesis, the number of cell surface *del 8* E-cadherin molecules compared to *wt* E-cadherin was quantified in MDA-MB-435S cells by flow cytometry using indirect immunofluorescence analysis. The amount of mutant E-cadherin at the plasma membrane was found to be only half of the amount of the *wt* protein (Figure 3.9). In addition, there was no difference between the expression levels of wt and mutant E-cadherin (data not shown). Together, these results suggest that mutation of E-cadherin, namely the loss of exon 8, influences the internalization of E-cadherin and destabilizes the protein at the plasma membrane. This result was confirmed with an independent cell system. L929 fibroblasts previously

transfected with *wt* or *del 8* E-cadherin (Handschuh et al., 1999) were included in the experiment and led to the same result.
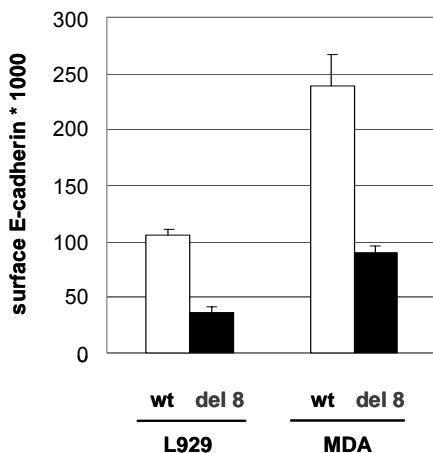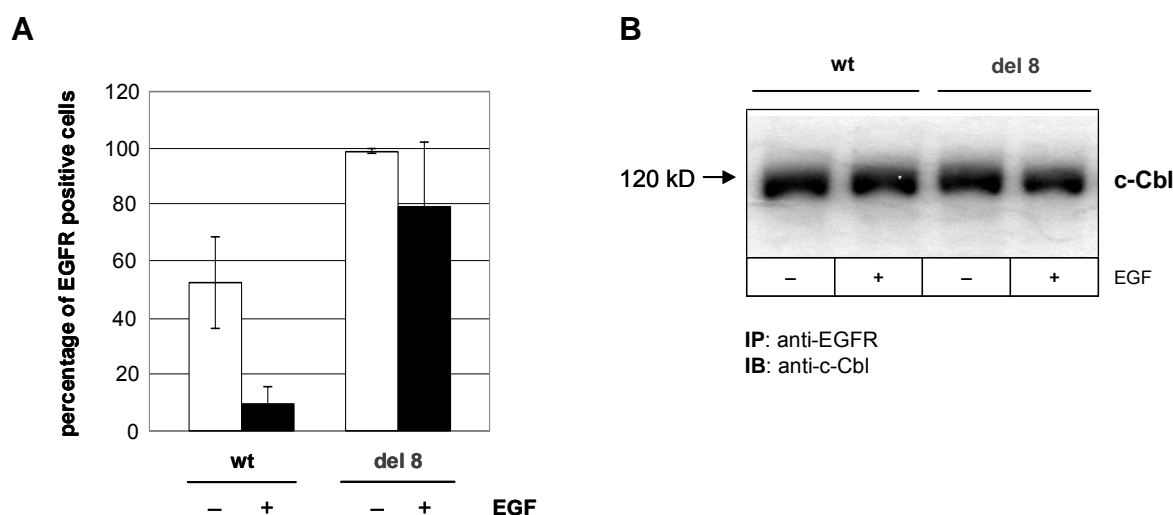

Figure 3.9: Surface localization of E-cadherin. The number of cell surface *del 8* E-cadherin versus *wt* E-cadherin was quantified in MDA-MB-435S cells and L929 cells by flow cytometry using indirect immunofluorescence analysis. Shown is the mean $\pm$ SD of two independent experiments. (Data generated by Dr. J. Mages)

3.1.10 Internalization of EGFR is affected by E-cadherin

Studies using EGFR fused to green fluorescent protein revealed that although EGF receptors are primarily localized at the cell surface, they constantly shuttle and recycle through the cell (Carter and Sorkin, 1998). After ligand binding, the rate of EGFR endocytosis is increased 5-10 fold. Colocalization of mutant E-cadherin and EGFR raised the question of whether mutant E-cadherin alters the localization and internalization of EGFR. FACS analysis revealed enhanced surface localization of EGFR in *del 8* compared to *wt* E-cadherin expressing cells (Figure 3.10 A). The mean percentage of EGFR positive cells was 52.4 % in *wt* and 98.7 % in *del 8* cells. After EGF stimulation, the internalization of EGFR was decreased in *del 8* cells. These data suggest that mutant E-cadherin influences the endocytosis of EGFR, which could have an effect on its activation.

EGFR phosphorylation creates a docking site to which the multifunctional adaptor protein and ubiquitin ligase c-Cbl is recruited (Grovdal et al., 2004). c-Cbl mediates ubiquitylation of EGFR which subsequently leads to the internalization of the receptor (Joazeiro et al., 1999). To study the contribution of c-Cbl to the observed differences in EGFR endocytosis in *wt* and *del 8* E-cadherin expressing cells, EGFR

immunoprecipitation was performed. Complex formation between EGFR and c-Cbl was identical in cells harbouring *wt* or *del 8* E-cadherin (Figure 3.10 B). Unexpectedly, the recruitment of c-Cbl was not affected by EGF stimulation. This result implies that the altered EGFR internalization in the presence of mutant E-cadherin is not reflected by changes in c-Cbl binding.

A

B

IP: anti-EGFR
IB: anti-c-Cbl

Figure 3.10: **Internalization of EGFR. (A)** *wt* and *del 8* E-cadherin expressing MDA-MB-435S cells were treated with EGF and the surface localization of EGFR was determined by FACS analysis. The mean percentage of EGFR positive cells $\pm$ SD of four independent experiments is shown. **(B)** Binding of Cbl to EGFR was studied in the presence of *wt* and *del 8* E-cadherin. Extracts of serum starved untreated and EGF stimulated cells were immunoprecipitated with anti-EGFR antibody followed by immunoblotting with anti-Cbl antibody. Shown is one representative of two independent experiments.

3.1.11 Expression and localization of E-cadherin and phosphorylated EGFR in gastric cancer

Besides the results obtained using the MDA-MB-435S cell culture model, the mechanism of EGFR activation by mutant E-cadherin was analysed in gastric tumours. In order to investigate the phosphorylation status of EGFR in gastric carcinomas in the presence of mutations of E-cadherin within the *hot spot* region comprising exons 8 and 9, 10 gastric adenocarcinomas of the diffuse type with exon 8 or 9 deletions were examined immunohistochemically (Table 3.1). By using the mutation-specific E-cad δ 8-1 and δ 9-1 antibodies (Figure 3.11), identification of mutant variants of E-cadherin in tumours was possible. Thus, DNA isolation from tumour tissue and subsequent sequencing was unnecessary.

Phosphorylated EGFR was detected in 8 of 10 cases with *del 8* or *del 9* E-cadherin mutations. Conversely, phosphorylated EGFR was detected only in 3 of 10 diffuse type and in 3 of 10 intestinal type gastric adenocarcinomas without *del 8* or *del 9* E-cadherin mutations (Tables 3.1 and 3.2). One tumour displaying strong membranous staining of *del 9* E-cadherin (Figure 3.12 A) and phosphorylated EGFR (at tyrosine residue 1086) (Figure 3.12 B) is shown as an example. In contrast, a gastric adenocarcinoma of the intestinal type showed strong wild-type E-cadherin staining (Figure 3.12 C) and complete absence of phosphorylated EGFR staining (Figure 3.12 D). To test whether enhanced EGFR activation was dependent on a dominant effect of the exon 8 or 9 mutations or whether it was also present in tumours which have lost E-cadherin due to other mechanisms, tumours where stratified according to the E-cadherin expression status (Table 3.3). Evidence was obtained that phosphorylated EGFR was present in 30 % of tumour cells independent of the presence or absence of E-cadherin. These results clearly indicate that enhanced EGFR activation observed in the presence of *del 8* or *del 9* E-cadherin is due to a dominant effect of the mutations.
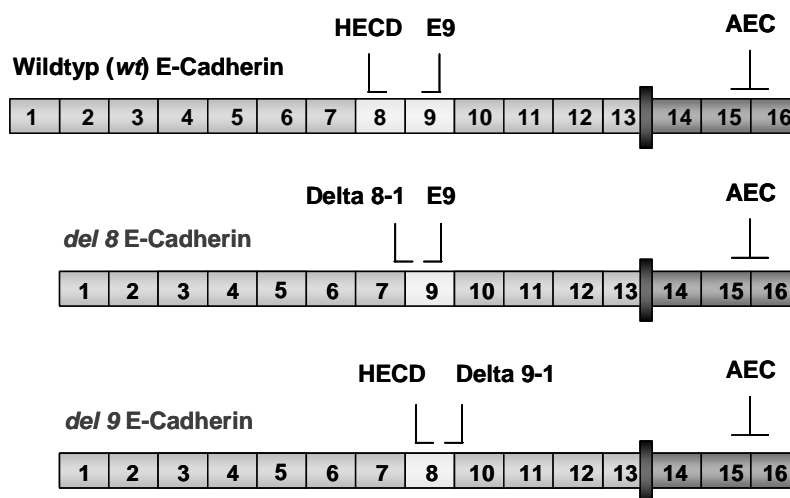
Figure 3.11: E-cadherin-specific antibodies. The mutation-specific antibodies Delta 8-1 and Delta 9-1 recognize *del 8* or *del 9* E-cadherin respectively.
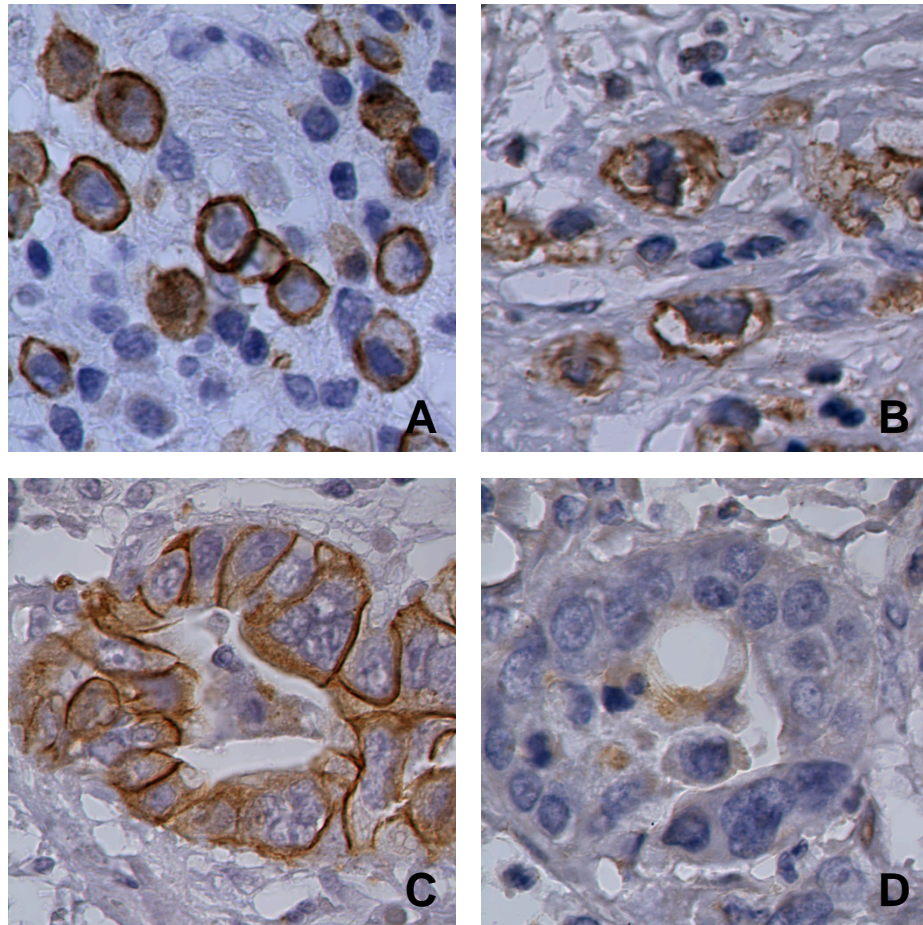
Figure 3.12: Immunohistochemical staining for E-cadherin and pEGFR in gastric adenocarcinoma.
Adenocarcinoma of the diffuse type displaying membranous staining of E-cadherin with deletion of exon 9 **(A)** and pEGFR phosphorylated at tyrosine residue 1086 **(B)**. Adenocarcinoma of the intestinal type as an example for strong E-cadherin staining **(C)** accompanied with complete absence of phosphorylated EGFR staining **(D)**.

Table 3.1*:* Expression of E-cadherin and pEGFR in gastric cancer

Patient	Histologic classification (Laurén)	*del 8* or *del 9* E-cadherin mutation	E-cadherin expression	pEGFR expression
1	Diffuse	*del 8*	+	+
2	Diffuse	*del 8*	+	+
3	Diffuse	*del 8*	+	+
4	Diffuse	*del 8*	+	-
5	Diffuse	*del 9*	+	+
6	Diffuse	*del 9*	+	-
7	Diffuse	*del 9*	+	+
8	Diffuse	*del 9*	+	+
9	Diffuse	*del 9*	+	+
10	Diffuse	*del 9*	+	+
11	Diffuse	-	-	-
12	Diffuse	-	-	-
13	Diffuse	-	-	+
14	Diffuse	-	-	+
15	Diffuse	-	-	-
16	Diffuse	-	-	-
17	Diffuse	-	-	-
18	Diffuse	-	+	+
19	Diffuse	-	+	-
20	Diffuse	-	+	-
21	Intestinal	-	-	-
22	Intestinal	-	-	-
23	Intestinal	-	-	+
24	Intestinal	-	+	-
25	Intestinal	-	+	-
26	Intestinal	-	+	+
27	Intestinal	-	+	+
28	Intestinal	-	+	-
29	Intestinal	-	+	-
30	Intestinal	-	+	-

Table 3.2: Summary of immunohistochemical data

Number of cases	Histologic classification (Laurén)	*del 8* or *del 9* E-cadherin mutation	E-cadherin expression (HECD-1 antibody)	pEGFR expression
10	Diffuse	*del 8:* 4 (40 %) *del 9:* 6 (60 %)	n.d.	8 (80 %)
10	Diffuse	-	3 (30 %)	3 (30 %)
10	Intestinal	-	7 (70 %)	3 (30 %)

n.d.: not determined

Table 3.3: pEGFR expression in diffuse and intestinal gastric cancer without *del 8* or *del 9* E-cadherin mutation

| Number of cases	Histologic classification (Laurén)	E-cadherin expression	pEGFR expression
10	Diffuse: 3 Intestinal: 7	10 (100 %)	3 (30 %)
10	Diffuse: 7 Intestinal: 3	0 (0 %)	3 (30 %)

n.d.: not determined

## 3.2 Mutant E-cadherin and its influence on further tyrosine kinases

Tyrosine phosphorylation has been previously implicated in the regulation of cadherin function. One mechanism by which tyrosine kinases can disrupt the cytoplasmic cell adhesion complex is by targeting E-cadherin for degradation. Otherwise functional adhesion junctions can affect the activity of receptor tyrosine kinases. As described in this study tumour-derived somatic E-cadherin mutations lead to enhanced EGFR phosphorylation. Thus, the question of whether mutant E-cadherin interferes with the activity of further tyrosine kinases was analysed.

3.2.1 Activation of hepatocyte growth factor receptor (Met) in MDA-MB-435S transfectants

A large number of studies show that hepatocyte growth factor / scatter factor (HGF/SF) and its receptor Met are frequently expressed in carcinomas. Dysregulation of HGF/SF and Met often correlates with poor prognosis (Birchmeier et al., 2003). Recent data indicate, that Met can interact with E-cadherin at the plasma membrane to form a signalling platform (Reshetnikova et al., 2007). To characterize the impact of tumour-derived mutant E-cadherin on Met activity, *wt* and mutant E-cadherin transfectants as well as mock transfected MDA-MB-435S cells at 40 % confluency were subjected to immunoblot analysis using an antibody which recognizes Met phosphorylated at tyrosines 1230, 1234 and/or 1235. Compared to mock cells, activation of Met was slightly increased in E-cadherin expressing cells (Figure 3.11). No significant differences in Met activity were detected in the presence of *wt* and mutant E-cadherin. In addition, the expression level of Met was equal in all transfectants (data not shown). These observations imply that E-cadherin with deletion of exon 8 or 9 do not change Met signalling.

A

B
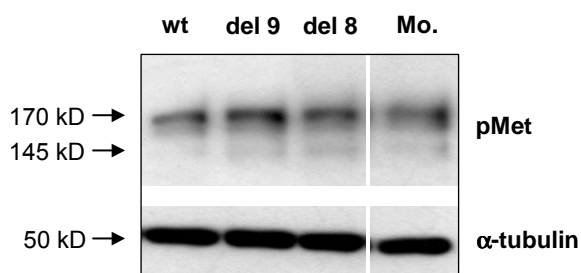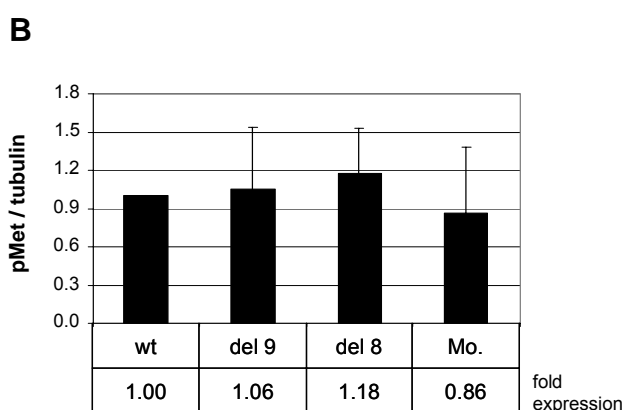
Figure 3.13: Activation of Met in MDA-MB-435S transfectants. (A) Serum starved *wt* and mutant (*del 9, del 8*) E-cadherin expressing MDA-MB-435S cells were treated with 20 ng/ml HGF for 5 min. Phosphorylation of Met receptor at Y1230, Y1234 and/or Y1235 was investigated by immunoblot analysis. **(B)** Densitometric analysis using Scion Image Software. Each bar represents the mean $\pm$ SD of three independent experiments. Mo.: mock transfected cells.

3.2.2 Mutant E-cadherin and the non-receptor tyrosine kinase Src

Src expression and/or activity are often elevated during epithelial cancer progression (Irby and Yeatman, 2000). While phosphorylation events by endogenous Src were shown to be required for the normal function of adherens junctions (AJs), phosphorylation of AJ components by oncogenic Src and by RTKs is generally associated with disruption of cell-cell adhesion, loss of epithelial differentiation and increased invasiveness (Behrens et al., 1993; Calautti et al., 1998). Given these opposed effects on cell adhesion the question came up if Src is modulated by expression of *wt* or mutant E-cadherin. Src activity is regulated by tyrosine phosphorylation at two sites with opposing effects. Phosphorylation at Y416 in the activation loop of the kinase domain upregulates enzyme activity whereas phosphorylation of Y527 in the carboxy-terminal tail renders the enzyme less active (Hunter, 1987). Exemplarily, Src activity was determined by immunoblot analysis in *wt* and *del 8* E-cadherin expressing cells. Therefore, a phospho-epitope specific antibody was applied that detects endogenous levels of Src only when phosphorylated at tyrosine 416 (Figure 3.12). It turned out that regulation of Src activity is not significantly different in the presence of *wt* or mutant E-cadherin, neither in EGF-stimulated nor in untreated transfectants.

A

B
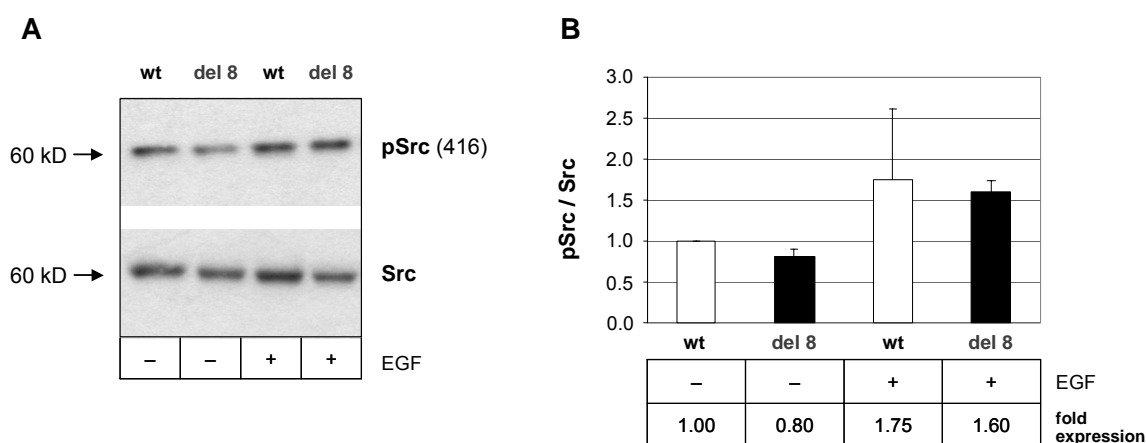
Figure 3.14: Src expression and activation. (A) Western blot analysis showing Src expression and phosphorylation level in serum starved untreated or EGF-stimulated *wt* and *del 8* E-cadherin expressing MDA-MB-435S cell. **(B)** Densitometric analysis using Scion Image Software. Each bar represents the mean $\pm$ SD of two independent experiments.

3.3 Matrix Metalloproteinase 3 (MMP-3)

Tumour progression is characterized by loss of cell adhesion and increase of invasion and metastasis. Degradation of the extracellular matrix by matrix metalloproteinases is necessary for tumour cell spread. Fuchs and colleagues revealed that E-cadherin mutations interfere with the MMP-3 suppressing function of E-cadherin. Additionally, MMP-3 has been identified to play a role in mutant E-cadherin-enhanced cell motility (Fuchs et al., 2005). Beside the characterization of the interplay of mutant E-cadherin and RTKs, one goal of this study was to determine the role of MMP-3 in the EGFR pathway.

3.3.1   Secreted MMP-3 is active in MDA-MB-435S-transfectants

It has been shown that expression of *wt* E-cadherin in MDA-MB-435S cells leads to downregulation of MMP-3 protein expression and secretion (Fuchs et al., 2005). Next, the question whether secreted MMP-3 possesses proteolytical activity was clarified by zymography analysis. Zymography is an electrophoretic technique based on SDS-PAGE, which includes a substrate copolymerised with a polyacrylamide gel for the detection of enzyme activity. Supernatant of *wt* and mutant E-cadherin (*del 8*, *del 9*) expressing cells was examined. Following electrophoresis, the polyacrylamide gel containing casein was incubated in substrate buffer to stimulate MMP-3 activity.

Subsequently, digestion of the substrate was determined. Degradation of casein by MMP-3 was enhanced in *del 9* E-cadherin cells compared to cells expressing *del 8* or *p8* E-cadherin (Figure 3.13 B). MMP-3 enzymatic activity was associated with a 45 kD band. *Wt* E-cadherin inhibited substrate degradation. Taken together, secreted MMP-3 zymogen is activated in MDA-MB-435 transfectants and the E-cadherin mutation status might influence MMP-3 activity.

A

B
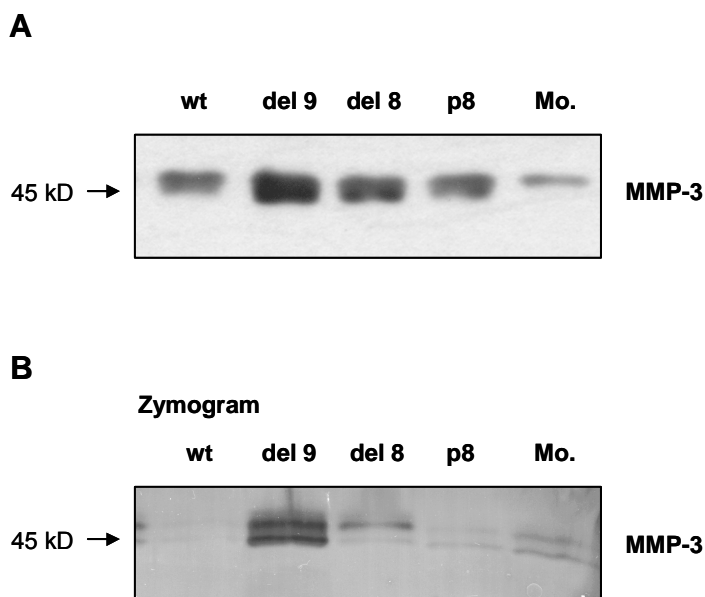
Figure 3.15: Zymography of MMP-3. (A) MMP-3 release into the medium of *wt* and mutant (*del 9, del 8, p8*) E-cadherin expressing MDA-MB-435S cells was determined by immunoblot analysis. **(B)** Zymography: Supernatant of *wt* and mutant (*del 9, del 8, p8*) E-cadherin cells were loaded onto a SDS-polyacrylamide gel containing casein. After electrophoresis, the gel was incubated in substrate buffer to activate MMP-3, stained with Comassie Blue, destained and photographed. MMP-3 enzymatic activity was associated with a 45-kD band where casein was degraded. Mo.: mock transfected cells.

3.3.2 Upregulation of MMP-3 in d*el 9* E-cadherin promotes cell invasion

Gastric carcinoma-derived E-cadherin mutations exhibit multiple effects on cell adhesion, motility, proliferation and tumourigenicity (Fricke et al., 2004; Fuchs et al., 2002; Handschuh et al., 1999; Kremer et al., 2003). So far, nothing is known about the influence of mutant E-cadherin on cell invasion. By using *Matrigel™ Invasion Chambers*, the invasive property of MDA-MB-435S transfectants was assessed *in vitro*. *Matrigel™ Invasion Chambers* consist of cell culture inserts containing an 8 µm pore size membrane with a thin layer of *Matrigel™* basement membrane matrix. The layer occludes the pores of the membrane, blocking non-invasive cells from migrating through the membrane. In contrast, invasive cells are able to detach themselves from and invade through the *Matrigel™* matrix and the pores.

Because *del 9* E-cadherin leads to the most elevated level of secreted MMP-3, this study was confined to the comparison of *wt* and *del 9* E-cadherin transfectants. In the presence of *del 9* E-cadherin, the number of invasive cells was upregulated compared to those cells expressing the *wt* molecule (Figure 3.14). Of note, the invasive behaviour of *del 9* cells could be reduced by application of the synthetic MMP inhibitor NNGH, thereby identifying MMP-3 as mediator of the effect. Hence, these data suggest a more invasive phenotype of *del 9* E-cadherin expressing cells that is caused by enhanced secretion of MMP-3.
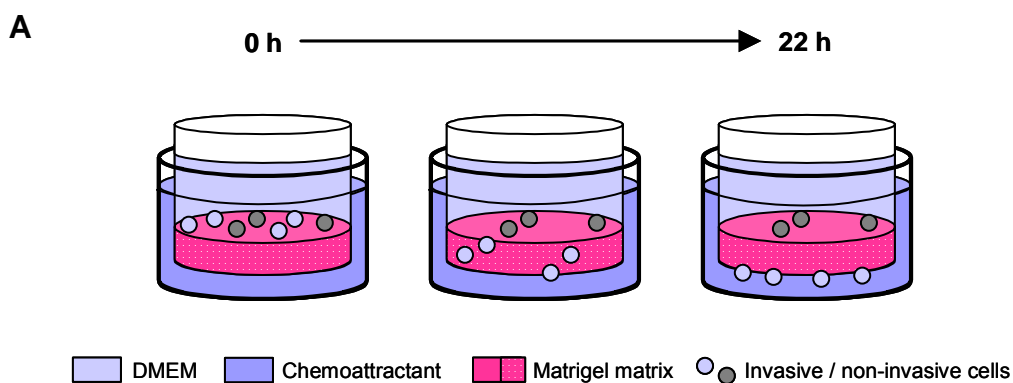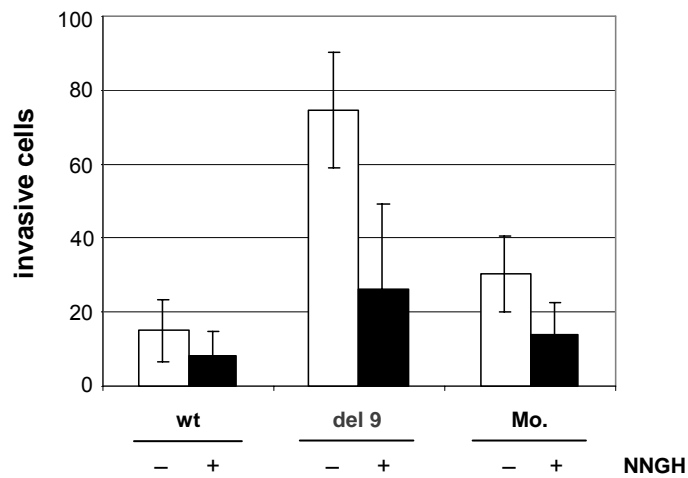
Figure 3.16: Enhanced cell invasion in *del 9* E-cadherin cells. (A) Principle of invasion assay. **(B)** *Wt* and *del 9* E-cadherin cells as well as mock transfected cells were seeded in *BioCoat™ Matrigel™ Invasion Chambers*. After 22 h invasive cells were determined by counting the cells that invaded through the *Matrigel™* matrix. Application of the MMP-3 inhibitor NNGH decreased the invasive behaviour of the cells. The average number of invasive cells ± SD from two independent experiments performed as triplicates is shown. Mo.: mock transfected cells.

4 Discussion

4.1 Crosstalk between mutant E-cadherin and EGFR

Mutations in the tumour suppressor gene E-cadherin and overexpression of the RTK EGFR are among the most frequent genetic alterations associated with gastric carcinoma (Becker et al., 1994; Gamboa-Dominguez et al., 2004). This is the first study of a functional relationship between EGFR and E-cadherin harbouring gastric carcinoma-derived somatic mutations. The investigated E-cadherin mutations were *in frame* deletions of exon 8 or 9, and a point mutation in exon 8, leading to partial loss of the adhesive function and up-regulation of cellular motility (Fuchs et al., 2002; Handschuh et al., 1999). EGFR activation by *del 8* E-cadherin was accompanied with enhanced binding of the adapter proteins Shc and Grb2 and an increased level of active Ras. In addition, Src activity was not altered in *del 8* cells. Src has been shown to act as a contributor to EGFR activation and/or as a signal transducer downstream of EGFR but might not be involved in mutant E-cadherin enhanced EGFR activation. Complex formation of *wt* and *del 8* E-cadherin was observed, indicating that deletion of exon 8 in the extracellular domain of E-cadherin still permits interaction of mutant E-cadherin with EGFR. Of note, association of *del 8* E-cadherin with EGFR was weaker compared to the *wt* protein. Reduced binding of mutant E-cadherin to EGFR in a multicomponent complex or reduced stability of the complex may enhance EGFR surface motility, thereby facilitating EGFR dimerization and activation. Moreover, reduced surface localization due to enhanced endocytosis of mutant E-cadherin compared to the *wt* protein was observed. In addition, the internalization of EGFR was decreased in response to EGF stimulation in cells expressing mutant E-cadherin, suggesting that mutation of E-cadherin also influences the endocytosis of EGFR. Finally, activation of EGFR in gastric carcinoma samples with mutant E-cadherin lacking exons 8 or 9 has been demonstrated.

4.1.1 Mechanism of EGFR activation by somatic E-cadherin mutations

The mechanism for ligand-induced dimerization of the extracellular domain of EGFR has been unravelled (Schlessinger, 2002). Formation of an asymmetric dimer with one kinase domain in the EGF-mediated dimer activating the other through an allosteric

mechanism has shed some light on the mechanism by which the receptor is activated (Zhang et al., 2006). There are several possible explanations for the increase in EGFR activation observed in this study in the presence of the E-cadherin mutation.

First, reduced complex formation between *del 8* E-cadherin and EGFR might be involved in the effect. EGFR colocalizes with E-cadherin to basolateral areas of polarized epithelial cells and both receptors are present in a multicomponent complex. E-cadherin has been shown to sequester EGFR by complex formation which leads to reduced receptor mobility and subsequent inhibition of receptor activation (Qian et al., 2004). Additionally, Qian and colleagues demonstrated that the sequences required for complex formation with EGFR are located within the extracellular region of E-cadherin. Although β-catenin can bind EGFR (Hoschuetzky et al., 1994) and is a substrate for this RTK, interaction between E-cadherin and EGFR occurred independently of the intracellular sites on E-cadherin that bind β-catenin or p120-catenin, which is another region for protein-protein interaction. Deletion of exon 8 of the E-cadherin gene is located within the *hot spot* region comprising exons 8 and 9, affecting the protein region connecting extracellular domains 2 and 3. This region seems to be the most critical domain in the process of tumour formation and progression, resulting both in loss of function (reduced cell-cell adhesion) and gain of function (increased motile behaviour) (Handschuh et al., 1999; Handschuh et al., 2001). The *del 8* mutation affects a putative calcium binding motif and may result in reduced binding of calcium. Since calcium binding is required for E-cadherin function and protease resistance (Leckband and Prakasam, 2006), the E-cadherin mutation might impair the overall structure and stability of the protein. A conformational change of E-cadherin might affect its capacity to bind to EGFR and abrogate EGFR regulation. In accordance with the presented results, a reduction in complex formation between EGFR and E-cadherin with germline mutations in the extracellular region (T340A, A634V) that abrogate cell adhesion was detected (Moutinho et al., 2008). These germline E-cadherin mutations were also associated with increased activation of EGFR. In contrast, intracellular E-cadherin germline mutations (P799R and V832M) had no detectable effect on the strength of binding in that study. Taken together, these results point to a crucial role of a mutation within the extracellular region of E-cadherin in complex formation with EGFR and activation of EGFR signalling. Reduced binding of mutant E-cadherin to EGFR might increase receptor mobility and facilitate receptor dimerization.

Second, the deletion of exon 8 changes the surface localization and endocytosis of E-cadherin, possibly contributing to the effect of the mutation on EGFR signalling. *del 8* E-cadherin is located in the cytoplasm, in the perinuclear region, and in lamellipodia, besides being present at the remaining cell-cell contacts that are formed despite the mutation (Luber et al., 2000). The amount of *del 8* E-cadherin residing at the plasma membrane was found to be only half of the amount of the wild-type protein. Reduced surface localization associated with enhanced internalization of mutant E-cadherin might influence the endocytosis of EGFR because both proteins colocalize (Hoschuetzky et al., 1994). Consistent with this hypothesis, endocytosis of EGFR has been shown in this study to be differently regulated in the presence of *wt* or mutant E-cadherin. *del 8* E-cadherin expressing cells exhibited enhanced surface localization of EGFR compared to *wt* E-cadherin cells. As expected from the literature (Carpenter and Cohen, 1976; Haigler et al., 1979), EGF stimulation led to subsequent internalization of EGFR in the presence of *wt* E-cadherin. Interestingly, EGFR endocytosis was decreased in response to EGF stimulation in cells expressing *del 8* E-cadherin resulting in stabilized EGFR at the membrane. Thus, mutant E-cadherin influences the internalization of EGFR which could have an effect on its activation and the downstream signalling.

Cbl is known to contribute to EGFR trafficking. After EGF-dependent receptor activation, Cbl is recruited to EGFR indirectly by Grb2 (Meisner and Czech, 1995) or directly to phospho-tyrosine 1045 (Grovdal et al., 2004). This enables several Cbl molecules to be recruited to active EGFR complexes and to mediate ubiquitylation – and subsequent internalization – of the oligomeric receptors. Whether ubiquitylation is instrumental for receptor internalization has been conversely discussed (Holler and Dikic, 2004).

In *wt* and *del 8* E-cadherin transfectants, Cbl was equally recruited to EGFR and did not reflect the observed alterations in endocytosis. Until recently, studies have focused on the role of EGFR tyrosine kinase activity and phosphorylation of the EGFR carboxyl terminus and other downstream proteins in regulating EGFR internalization. Now, Wang and colleagues showed that EGF-induced receptor dimerization is the event that triggers EGFR internalization suggesting that EGFR kinase activation and the consequent downstream signalling events are not required (Wang et al., 2005). These results point to a secondary role of Cbl and further downstream molecules in EGFR endocytosis and would allow differently regulated EGFR trafficking in *wt* and *del 8*

E-cadherin cells, even though Cbl is equally recruited to the receptor. Hence, influencing EGFR dimerization rather than recruitment of adaptor molecules would strongly affect receptor internalization.

Taken together, these data suggest that the two mechanisms, reduced complex formation between mutant E-cadherin and EGFR as well as reduced surface localization of E-cadherin combined with decreased internalization of EGFR contribute to enhanced EGFR activation observed in the presence of the E-cadherin mutation.

4.1.2   EGFR activation in tumours

Modulation of EGFR signalling has frequently been observed in neoplastic transformation. Mechanisms leading to constitutive activation of EGFR include increased production of ligands, elevated levels of EGFR protein, EGFR mutations and defective down-regulation of EGFR (Zandi et al., 2007). The functional role of EGFR in cancer cells is also affected by communication with other cell surface receptors, e.g. other ErbB family members or RTKs, cell adhesion molecules or cytokine receptors. In this study it has been shown that extracellular mutation of E-cadherin contributes to enhanced EGFR activation, thereby presenting a novel mechanism of oncogenic EGFR signalling.

In various types of cancer, growth factors of the EGF-family are found co-expressed with EGFR. These growth factors can activate EGFR in an autocrine/paracrine manner, leading to uncontrolled proliferation (Cai et al., 1999; Cohen et al., 1994; Umekita et al., 2000). However, cellular transformation seems to be dependent on both the level of ligand and receptor (Rosenthal et al., 1986). Increased EGFR has been found to correlate with decreased survival in diverse cancer types. Amplification of the EGFR gene, elevated activity of the EGFR promoter, and deregulation at the translational and post-translational levels are mechanisms by which the amount of EGFR can be increased in tumour cells. EGFR overexpression has been described among others in lung, colon, breast, brain and also in gastric cancer (Gullick, 1991; Libermann et al., 1985). EGFR mutations cluster in the intracellular kinase domain of EGFR (exon 18-21) and cause ligand-independent receptor activation. Until recently, EGFR mutations have not been observed in primary gastric carcinomas. Moutinho and colleagues identified somatic EGFR mutations located in the kinase domain of EGFR. Gastric carcinoma harbouring these EGFR mutations exhibits a significant increase in tumour size (Moutinho et al., 2008). In the present study, another way of EGFR activation in

gastric tumours has been shown, namely by E-cadherin mutations. pEGFR was detected in 80 % of gastric adenocarcinomas of the diffuse type with exon 8 or 9 deletions. In contrast, pEGFR was detected only in 30 % of diffuse type or intestinal type gastric Adenocarcinoma, independent of the presence of E-cadherin without *del 8* or *del 9* mutations. Thus, in the analysed tumour series, lack of E-cadherin expression did not automatically result in EGFR activation. These findings indicate that EGFR activation observed in the presence of *del* 8 or *del* 9 E-cadherin is due to a dominant effect of the mutations. Loss of E-cadherin due to other mechanisms might also influence the EGFR pathway. To further investigate this aspect, a larger tumour series should be analysed.

4.1.3   EGFR as a target for gastric cancer therapy?

Despite improvements in both surgical techniques and radio-chemotherapy regimens, gastric cancer remains a great therapeutic challenge. Complete removal of the tumour by surgery is the only available treatment to provide cure in localized, non-metastatic gastric cancer (Roukos and Kappas, 2005). However, the high rate of recurrence and metastatic spread after surgical resection have provided a powerful rationale for the use of integrated treatments, basically represented by neoadjuvant and adjuvant radio-chemotherapy. Despite encouraging results, these aggressive multimodal treatments have been highly questioned because of eligibility criteria, different surgical treatments, and true benefit (Hartgrink et al., 2004; Hundahl and Wanebo, 2005; Leong, 2004; Stahl, 2004). Thus, controversy still exists about the best management for gastric cancer.

Development of treatment strategies to prevent recurrences is the main goal of current research. Knowledge of molecular pathways determining the behaviour of individual gastric tumours seems to be crucial for therapeutic decisions. EGFR is highly expressed in gastric cancer (Kim et al., 2008; Gamboa-Dominguez et al., 2004). While in some human tumours a large body of clinical data has shown that selective anti-EGFR targeted therapies have potent anti-tumour activity alone and in combination with certain cytotoxic treatments (Baselga and Arteaga, 2005; Mendelsohn and Baselga, 2003), in gastric cancer the efficacy of this targeted therapy is going to be evaluated in a phase III study (http://clinicaltrials.gov/ct2/show/NCT00678535) only now. Nevertheless, EGFR inhibition in gastric cancer patients represents a feasible therapeutic option. A subgroup of patients with non-small cell lung cancer have specific mutations in the EGFR gene, which correlate with a rapid and often dramatic clinical

response to the TKI erlotinib, which targets EGFR (Lynch et al., 2004). These mutations lead to increased EGFR signalling and confer susceptibility to the inhibitor. Screening for such mutations in lung cancer may identify patients who will respond to gefitinib.

In accordance with these findings, the present study could provide a method for screening gastric cancer patients to detect E-cadherin mutations (which provoke enhanced EGFR activation) in order to identify those group of patients that would be responsive to treatment with an EGFR antagonist.

4.2 Src-independent changes in cell adhesion and motility

AJs are not static. Even in a confluent epithelial monolayer, there is a continual endocytosis and recycling of cadherin molecules, albeit to a lesser extent than in sub-confluent cells (Le et al., 1999). As described before, tumour-derived somatic mutation of E-cadherin results in decreased surface localization associated with enhanced internalization compared to the *wt* receptor. The regulation of AJ formation and stability may be influenced both by signals acting on junctional components, and by signals originating from the nascent junction itself. Tyrosine phosphorylation of various catenins by receptor and non-receptor tyrosine kinases has been associated with both positive and negative changes in adhesion.

Src family kinases have been shown to co-localize with E-cadherin at sites of cell-cell adhesion in non-migrating epithelial cell (Calautti et al., 1998; Owens et al., 2000). Low levels of Src family kinases are required in normal tissue to keep the integrity of the epithelium. The Src SH domains contribute positively to the assembly of epithelial cell-cell contact (Calautti et al., 1998). During cancer progression, Src expression and/or activity are often elevated and affect the cellular adhesion that control cell migration and invasion (Irby and Yeatman, 2000). High-level kinase activity was shown to negatively regulate the ability of cells to form cadherin-mediated cell-cell contacts by activating integrins and FAK signalling (Avizienyte et al., 2002). Src-induced deregulation of cadherin-based cell-cell adhesion also occurs via initiation of an endocytic pathway that results in internalization of E-cadherin.

As determined in this study, Src expression and activity are not significantly altered in *wt* and *del 8* E-cadherin transfectants. Only a faint lowering of Src activity was observed in *del 8* E-cadherin cells. Therefore, Src activity might not be involved directly

in mutant E-cadherin decreased cell-cell adhesion and increased *del 8* E-cadherin internalization. This finding would rather support Src as a positive contributor to *wt* E-cadherin mediated cell-cell adhesion. So far, one cannot define the impact of Src on MDA-MB-435S transfectants. The question of whether Src positively influences *wt* E-cadherin cell-cell adhesion remains to be solved. Mutant E-cadherin influences downstream signalling which in turn affects AJs and regulate their stability. The results presented in this study underline a direct role of *del 8* E-cadherin in reduced cell-cell adhesion, that is the influence of the structural modification of the adhesion molecule on cell-cell contact formation. Indirect influence via modulating downstream signalling molecules which affect again cell-cell adhesion seams to play a subsidiary role in the case of Src.

In addition, Src interacts with- and participates in signalling from RTKs (Bromann et al., 2004). The synergy between Src and EGFR is complex and bidirectional and raises the question of whether Src contributes to mutant E-cadherin enhanced cell motility. Equally expressed and activated Src provided no indication for its participation in differently regulated EGFR signalling and enhanced cell motility in mutant E-cadherin expressing cells.

4.3 MMP-3 in cell invasion and motility

Matrix metalloproteinases (MMPs) play an important role in the invasion and metastasis of malignant cells. In previous studies, MMP-3 expression and secretion has been shown to be upregulated in mutant E-cadherin expressing MDA-MB-435S cells (Fuchs et al., 2005). The mutations interfered with the MMP-3 suppressive function of *wt* E-cadherin and promoted cell motility. Now, in this study the secreted MMP-3 zymogen was defined as an active enzyme. Because degradation and remodelling of the extracellular matrix by MMPs is necessary for tumour cell spread, the contribution of MMP-3 in cell invasion was investigated in *wt* and mutant E-cadherin cells. Expression of *del 9* E-cadherin correlated with the highest MMP-3 level and exhibited the most invasive phenotype compared to cells harbouring other E-cadherin mutations (unpublished data by Joëlle Deplazes) or the *wt* receptor. By using the synthetic MMP inhibitor NNGH, cell invasion could be blocked, thereby identifying MMP-3 as a mediator of the effect. These observations imply that *wt* E-cadherin downregulates MMP-3 to prevent tumour invasion and that the invasion suppressor

function seems to be impaired by E-cadherin mutations. The mechanism which regulates the functional interaction between MMP-3 and E-cadherin is unclear.

β-catenin exists in different pools within the cell. Mainly, it contributes to the cytoplasmic cell adhesion complex as a binding partner of E-cadherin but β-catenin also acts as a transcription factor and mediator of the Wnt signalling pathway. Somatic E-cadherin mutations induce a cytoplasmic and perinuclear localization of β-catenin compared to the almost exclusive membrane localization in cells expressing *wt* E-cadherin (Luber et al., 2000). In the nucleus, β-catenin regulates the expression of a wide variety of target genes containing T-cell factor/lymphoid-enhancer factor (TCF/LEF) binding sites. Some MMPs have been shown to be regulated by β-catenin (Brabletz et al., 1999). Whether MMP-3 is among them has not yet been resolved, but MMP-3 exhibits a potential TCF4 binding site within its promoter region (Brabletz et al., 1999). As β-catenin is partly released from the membrane and relocated to the nucleus in the presence of mutant E-cadherin, this pool of β-catenin could be responsible for the transcriptional upregulation of MMP-3. Thus, β-catenin might constitute a link between mutant E-cadherin and increased MMP-3 expression. Cell fractionation experiments indicated that β-catenin is present in the nucleus in MDA-MB-435S transfectants (preliminary data), but the transcriptional regulation of MMP-3 by β-catenin remains to be investigated.

MMPs have, in addition to their ability to degrade matrix proteins and enhance cell motility, the capacity to cleave ectodomains of certain transmembrane proteins. Recently, MMP-3 has been shown to cleave the extracellular domain of E-cadherin, thereby producing an 80 kDa soluble E-cadherin fragment (Lochter et al., 1997; Noe et al., 2001). The remaining membrane-bound part of E-cadherin is subsequently proteolytically degraded (Ito et al., 1999), which results in the translocation of β-catenin from the cell membrane to the cytoplasm and in the loss of E-cadherin-mediated cell adhesion (Noe et al., 2001). In the present study, it has been verified that the high amount of secreted MMP-3 in mutant E-cadherin expressing cells is in its active conformation and would be able to degrade E-cadherin. Nevertheless, no soluble E-cadherin fragment was detected in MDA-MB-435S transfectants (unpublished data by Margit Fuchs), which suggests that in this model, the motility enhancement driven by MMP-3 is not dependent on E-cadherin ectodomain cleavage.
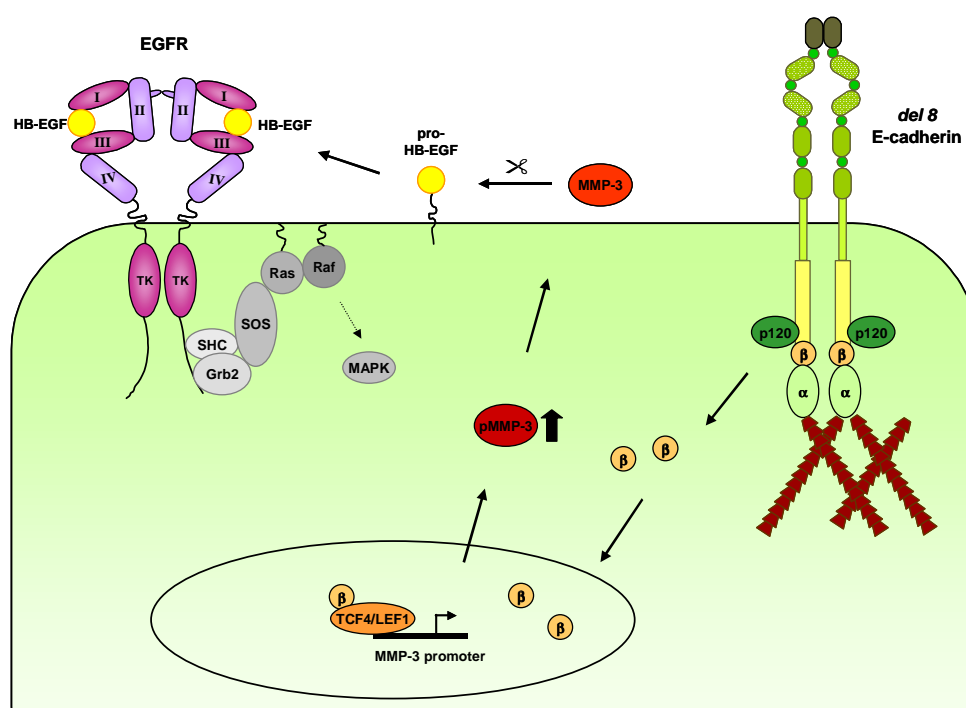
4.3.1   An integrated model for mutant E-cadherin enhanced cell motility

As already mentioned, both enhanced EGFR activation and upregulated MMP-3 expression and secretion contribute to *del* 8 E-cadherin enhanced cell motility. Can these independently investigated results be combined in one model, or do they represent independent processes within the cell?

Firstly, β-catenin might represent a link between E-cadherin and MMP-3. Secondly, growth factors of the EGF-family are synthesized as membrane-anchored precursors that are cleaved to release the mature soluble growth factor in a process designated ectodomain shedding (Suzuki et al., 1997). Among others, MMPs are responsible for this processing event. Activation of the EGFR ligand HB-EGF by MMP-3 has already been reported *in vitro* and *in vivo* (Suzuki et al., 1997) and could be involved in EGFR signalling in MDA-MB-435S transfectants. Recombinant HB-EGF caused elevated levels of activated EGFR in *del 8* E-cadherin expressing MDA-MB-435S cells compared to cells harbouring the *wt* receptor (date not shown). Thirdly, ligand-dependent EGFR activation provokes enhanced MMP-3 expression (Hosono et al., 1996; Unemori et al., 1994).

In summary, this model postulates that increased MMP-3 in *del 8* E-cadherin expressing cells is mediated by β-catenin and triggers cleavage of growth factors like HB-EGF, which could be a further explanation for enhanced activation of EGFR. HB-EGF processing by MMP-3 would increase the availability of the potent growth factor that has been shown to be involved in physiological and pathological processes. HB-EGF could activate EGFR signalling and consequently participate in enhanced cell motility. Previously, MMP-3 has been demonstrated to increase the availability of basic fibroblast growth factor (bFGF) by releasing it from basement membrane perlecan, which represents a major storage site for this growth factor in the blood vessel wall (Whitelock et al., 1996). Furthermore, EGF and TGF-α have been shown to augment MMP-3 production (Hosono et al., 1996; Unemori et al., 1994), suggesting the possible existence of an autocrine amplification loop wherein EGF-like growth factors increase MMP-3 levels, in turn increasing secreted HB-EGF levels.

Figure 4.1: Both enhanced EGFR activation and MMP-3 secretion contribute to *del 8* E-cadherin increased cell motility. Shown is a model which integrates both results and suggests that unbound β-catenin translocates to the nucleus and upregulates MMP-3 expression in *del 8* E-cadherin cells. MMP-3 cleaves pro-HB-EGF at the membrane to release the mature growth factor that may contribute to enhanced EGFR activation.

Of note, the model described above cannot completely be adapted to *del 9* E-cadherin cells. *del 9* E-cadherin promotes enhanced cell motility and invasion, as well as increased MMP-3 expression. In contrast to *del 8* and *p8* E-cadherin, EGFR activation was not upregulated in MDA-MB-435S cells expressing *del 9* E-cadherin compared to *wt* E-cadherin cells, even so levels of pEGFR could be detected in gastric tumours harbouring a *del 9* E-cadherin mutation. This observation suggests that E-cadherin mutations yield a similar output, i.e. enhanced cell motility and invasion, via different or overlapping mechanisms. Thus, tumour-derived E-cadherin mutations could represent an example for biological degeneracy which is the ability of structurally different elements to perform the same function or cause the same output (Edelman and Gally, 2001). This term previously was used in immunology to refer to the ability of different antibodies to bind to the same antigen (Edekman, 1974). Now, there is increasing evidence that degeneracy is a ubiquitous property of biological systems at all levels of

organization, e.g. various components of signalling pathways provide multiple examples of degeneracy (Edelman and Gally, 2001).

del 8 and *del 9* E-cadherin are structurally different and exert different effects on EGFR but both mutations provoke enhanced cell motility and invasion. One could argue that these mutations induce distinct conformational changes of E-cadherin. It is possible that complex formation of E-cadherin and EGFR is less affected by deletion of exon 9 then by deletion of exon 8, but this needs to be clarified. Taken together, the analysed E-cadherin mutations might use different mechanisms to trigger cell motility (EGFR signalling) and/or overlapping mechanisms (β-catenin).

4.4 Significance of EGFR activation by E-cadherin mutations for tumour progression

The EGFR signalling pathway is one of the most important networks that regulate cell proliferation, differentiation, survival and motility. Enhanced signalling of EGFR due to its overexpression is well known in several carcinomas, including gastric carcinoma (Gamboa-Dominguez et al., 2004). In the present study, a novel mechanism of regulating EGFR activation has been described. It has been shown that an extracellular mutation of E-cadherin contributes to the frequently observed activation of EGFR in tumours and gives one explanation for the motile and invasive behaviour of tumour cells with E-cadherin mutations.

4.5 Outlook

Using a severe combined immunodeficiency (SCID) mouse model, it has been shown that tumour-associated E-cadherin mutations influence tumourigenicity and metastasis (Kremer et al., 2003). Current investigations are evaluating the therapeutic benefit of the EGFR inhibitor cetuximab for the treatment of tumours with E-cadherin mutations in this SCID mouse xenotransplantation model. The aim of the experimental set-up is to gain insight into the efficacy of EGFR antagonists in tumours harbouring an E-cadherin mutation with regard to inhibition of tumour and metastasis formation *in vivo*.

Furthermore, analyses are planned to elucidate the mechanism of EGFR activation by mutant E-cadherin in more detail. The interaction between *wt* or mutant E-cadherin and EGFR will be characterised by FRET analysis. To determine potential changes in microdomain localization of both molecules, lipid rafts will be isolated. Finally, the influence of mutant E-cadherin on EGFR mobility in the plasma membrane will be specified using FRAP technique.

5 Summary

For the past few decades, gastric cancer mortality has decreased markedly in most areas of the world. However, gastric cancer remains a disease of poor prognosis and high mortality, second only to lung cancer as the leading cause of cancer-related deaths worldwide. In spite of intense interest and extensive investigations, its prognosis has not been improved significantly in recent years.

Gastric cancer is a product of multiple genetic and epigenetic alterations that transform normal gastric epithelial cells into malignant neoplasms. These alterations appear to arise in the permissive setting of genomic instability and, in general, activate oncogenes or silence tumour suppressor genes. Mutations of the tumour suppressor E-cadherin and overexpression of the receptor tyrosine kinase EGFR are among the most frequent genetic alterations associated with diffuse-type gastric carcinoma and initiate a more aggressive disease.

The E-cadherin/catenin complex is a calcium-dependent cell-cell adhesion molecule whose function is critical to the integrity of the adhesion junction and which plays a role in the establishment and maintenance of normal epithelial morphology and differentiation. Loss of E-cadherin-mediated adhesion appears to be a fundamental aspect of the neoplastic phenotype. E-cadherin was originally viewed exclusively as a structural protein mediating cell-cell adhesion. More recently, it has been shown that E-cadherin participates in signal transduction pathways.

Expression of gastric carcinoma-derived E-cadherin variants in MDA-MB-435S cells causes reduced adhesion and increased cell motility. By inhibiting EGFR, this receptor was identified to be involved in mutant E-cadherin enhanced cell motility. In the present study, the molecular mechanism linking E-cadherin mutations and the EGFR signalling pathway was explored. Somatic mutation of E-cadherin was associated with increased activation of EGFR followed by enhanced recruitment of the downstream acting signalling components Grb2 and Shc, and activation of Ras. Reduced complex formation of mutant E-cadherin with EGFR was observed compared to *wt* E-cadherin. Decreased binding of mutant E-cadherin to EGFR in a multicomponent complex or reduced stability of the complex may enhance EGFR surface motility, thereby facilitating EGFR dimerization and activation. Furthermore, reduced surface localization

due to enhanced internalization of mutant E-cadherin compared to the *wt* protein was observed. The internalization of EGFR was decreased in response to EGF stimulation in cells expressing mutant E-cadherin, suggesting that mutation of E-cadherin also influences the endocytosis of EGFR. In addition to the cell culture model, increased activation of EGFR in gastric carcinoma samples with mutant E-cadherin lacking exons 8 or 9 was demonstrated.

Closing, the functional link between mutant E-cadherin, EGFR and matrix metalloproteinase 3 was analysed. Like EGFR, MMP-3 contributes to enhanced motility in mutant E-cadherin expressing cells. In the present study, the participation of MMP-3 in cell invasion was identified. The enhanced invasive property of *del 9* E-cadherin expressing cells could be blocked by the MMP inhibitor NNGH. Additionally, it was shown that the secreted zymogen, which is increased in mutant E-cadherin cells, is an active enzyme. Thus, MMP-3 might process membrane-anchored growth factor precursors of the EGF-family and thereby influencing EGFR signalling.

In summary, activation of EGFR by mutant E-cadherin was described as a novel mechanism in tumour cells that explains the enhanced motility of tumour cells in the presence of an extracellular mutation of E-cadherin.

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7 Abbreviations

AA	amino acid
mAb/pAb	monoclonal/polyclonal antibody
ABC	antibody binding capacity
ACAL	antibody capture affinity ligand
AR	amphiregulin
AJ	adherens junction
APC	adenomatous polyposis coli
APS	ammonium persulfate
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BTC	betacellulin
BSA	bovine serum albumin
° C	degree Celsius
CCC	cytoplasmic cell-adhesion complex
del 8/del 9	deletion of exon 8 or 9
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTAF	dichlorotriazinylaminofluorescein
dNTP	deoxynucleotide triphosphate
E-cadherin	epithelial (E)-cadherin
EC-/CAD domain	extracellular E-cadherin domain
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol tetraacetic acid
EMT	epithelial-to-mesenchyme transition
EPR	epiregulin
ErbB	avian erythroblastosis oncogene B
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate

FRAP	fluorescence recovery after photobleaching
FRET	fluorescence resonance energy transfer
g	gram
g	earth's gravity
GDP	guanosine diphosphate
Grb2	growth factor receptor binding protein 2
GST	glutathion S-transferase
GTP	guanosine triphosphate
h	hour
HB-EGF	heparin-binding growth factor
HDGC	hereditary diffuse gastric carcinoma
HGF	hepatocyte growth factor
HNPCC	hereditary nonpolyposis colorectal cancer
HRP	horseradish peroxidase
IB	immunoblot /Western blot
IHC	immunohistochemistry
IP	immunoprecipitation
kD	kiloDalton
l	litre
L-CAM	liver cell adhesion molecule
LOH	loss of heterozygosity
NSCLC	non-smal cell lung cancer
M	molar
MAPK	mitogen-activated protein kinase
min	minute
Met-/HGF receptor	hepatocyte growth factor receptor
MMP	matrix metalloproteinase
MSI	microsatellite instability
μ	micro
NRG	neuregulin
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase

PKC	protein kinase C
PLCγ	phospholipase C-γ
PMSF	phenylmethylsulphonyl fluoride
PTB domain	phosphotyrosine binding domain
PVDF	polyvinylidene fluoride
RBD	Raf1 binding domain
rpm	revolutions per minute
RT	room temperature
RTK	receptor tyrosine kinase
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulfate
sec	second
SH2 domain	Src homology 2 domain
Shc	Src homologues and collagen protein
SNP	single nucleotide polymorphism
SOS	son-of-sevenless protein
STAT	signal transducer and activator of transcription
TBS	tris buffered saline
TCF/LEF	T-cell factor/lymphoid-enhancer factor
TEMED	tetramethylethylenediamine
TGF-α	transforming growth factor-α
TKI	small-molecule tyrosine-kinase inhibitor
Tris	tris(hydroxymethyl)aminomethane
U	enzyme activity unit
V	volt
% v/v	volume-volume percentage
wt	wild-type
% w/v	mass-volume percentage

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