

Epithelial to mesenchymal transition, cell surface receptors activation and intracellular communications in cancer metastasis

Review Article

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Abbreviations: amphiregulin, (AR); epiregulin, (EPR); epithelial-to-mesenchymal transition, (EMT); farnesyltransferase inhibitors, (FTIs); heparin-binding EGF, (HB-EGF); immunoglobulin, (Ig); insulin receptor, (IR); integrin-linked kinase, (ILK); IR substrate 1, (IRS-1); mitogen-activated protein kinase, (MAPK); phosphatidylinositol 3- kinase, (PI3 K); phospholipase C, (PLC); PI-3 kinase, (PI-3K); Rho-kinase, (ROCK); signal transducer and activator of transcription 3, (STAT3); signal transducers and activators of transcription, (STATs); Src family kinases, (SFKs); transforming growth factor- β , (TGF- β); transmembrane serine/threonine kinases, the type I and type II receptors, (T_{RI} and T_{RII})

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Summary

During the developmental cycle of a mammary gland, many properties that are associated with breast cancer are also displayed. The stromal factors necessary for mammary growth will either promote or protect against breast cancer. The epithelial-to-mesenchymal transition (EMT) is a developmental mechanism of crucial importance because it establishes the body plan in many multicellular organisms. Several transduction pathways controlling the various steps of this morphological transition have been identified by molecular analyses in both cell lines and *in vivo*. The newly formed mesenchymal cells can exhibit locomotory and invasive phenotypes, suggesting that EMTs may contribute to the progression of carcinomas. The genetic basis of tumorigenesis varies greatly between cancer types; however, the cellular and molecular steps required for metastasis are similar in all cancer types. This review will explore the connection between the EMT process and its involvement in the initiation and progression of breast cancer. Recently, there have been advances made in the understanding of molecular mechanisms that govern this lethal metastatic progression. New therapeutic approaches show promise because they target this process; however, before drug development can become successful, significant gaps in the basic knowledge of EMT processes and its molecular mechanisms must be met.

I. Introduction

A. The clinical dilemma

Most human tumors are of epithelial origin (carcinomas) and metastasis from such tumors lead to >80% of all cancer deaths (Hanahan and Weinberg, 2000; Greenlee et al, 2001). Unfortunately, the mechanisms involved in local invasion and metastasis are not fully understood. Thus, prognosis for a patient who is diagnosed with advanced invasive or metastatic disease has not improved in the past few decades (Sporn, 1997). The classical metastatic cascade encompasses intravasation by tumor cells, their circulation in lymph and blood vascular

systems, arrest in distant organs, extravasation and growth into metastatic foci (Herlyn and Malkowicz, 1991; Woodhouse et al, 1997). Most if not all of these events require plasticity from tumor cells that is demonstrated by their ability to adopt a variety of phenotypes (Sood et al, 2001, 2002).

B. EMT in normal development and cancer

Normal tissue homeostasis is maintained between epithelial cells and their microenvironment that may include fibroblasts, endothelial and immunocompetent

cells and the extracellular matrix (Nieto et al, 1994; Nieto, 2001). During malignant transformation and progression, there are (however deregulated) reciprocal and conspirational interactions between the neoplastic cells and the adjacent stromal cells (Bissell and Radisky, 2001; Wiseman and Werb, 2002). Although the genetic basis of tumorigenesis may vary greatly between different cancer types, the cellular and molecular steps required for metastasis are generally similar for all solid tumor cells (Woodhouse et al, 1997; Liotta and Kohn, 2003). Not surprisingly, the molecular mechanisms that propel invasive growth and metastasis are also found in embryonic development, but to a different extent. The perception of cancer has changed from viewing it just in its connection to genes to seeing it as a complex tissue resulting from disrupted organ homeostasis (Bogenrieder and Herlyn, 2002; Wiseman and Werb, 2002).

During development of even the most primitive species, remodeling of a simple epithelium by delamination, intercalation, invagination, evagination, branching, or cavitation of an aggregate of cells generates two or more layered epithelium (Hay et al, 1995; Markwald et al, 1996; Thiery, 2002). Some of these processes require reversible or in some instances irreversible conversion of epithelial cells into mesenchymal cells (EMT) (Birchmeier et al, 1996). EMT is defined as the acquisition of epithelium cells of fibroblastoid, migratory phenotype accompanied by profound changes in gene expression towards a mesenchymal program. It also includes the cell's ability to digest and transmigrate through basement membranes and heterologous tissues (Rudland et al, 1985). Parallels between EMT in development and in breast and other tumors progression have already been described. For instance, intra-tumor and inter-tumor heterogeneity of human breast cancer is considered as differentiation repertoires available to the neoplastic cells in response to the tumor microenvironment, including reversion to a 'normal' phenotype (Bissell et al, 1999) and not a consequence of phenotypic drifting due to genetic instability.

The EMT concept provides a new way to identify genes that are seen in the progression of carcinoma towards dedifferentiated and more malignant states (Gilles and Thompson 1996; Petersen et al, 1998; Thiery and Chopin, 1999; Boyer et al, 2000). In examining the cellular and developmental biology of EMTs, researches may gain insight into the mechanisms of tumor progression (Rønnov-Jessen et al, 1996). Several signaling pathways have been discovered to have a connection with EMTs. A more widespread knowledge of these pathways and the genes involved may be of great value to improve our understanding of breast cancer. This may allow doctors to provide a more reliable prognosis/therapies for their patients. Before an analysis of the data relevant to EMT can be examined, the major pathway initiating and maintaining it "the adherins pathway", must first be explained.

II. The adherins pathway

E-cadherin mediate homophilic interactions by

forming adhesive bonds between one or several immunoglobulin (Ig) domains in its extracellular region and by them connecting to actin microfilaments indirectly via β - and γ -catenin in the cytoplasm (Stockinger et al, 2001). The de novo production of E-cadherin in normal and transformed mesenchymal cells can induce the formation of stable cell-cell contacts and the development of adherens junctions to promote the formation of desmosomes (Birchmeier and Behrens, 1994). Clinically, loss of heterozygosity at 16q22.1 is relatively frequent in breast carcinoma, implicating E-cadherin as a breast tumor suppressor gene. Numerous studies have reported a partial or complete loss of E-cadherin during carcinoma progression, which is connected to an unfavorable prognosis (Yoshiura et al, 1995; Perl et al, 1998; Cheng et al, 2001). This confirms that E-cadherin is a caretaker of the epithelial state. *In vitro*, lack of E-cadherin production correlates directly to the loss of epithelial phenotype (Birchmeier and Behrens, 1994; Cheng et al, 2001; Ilyas et al, 2000). While *in vivo*, E-cadherin is down regulated specifically at sites of EMT, such as gastrulation in *Drosophila* and in several vertebrates including the mouse (Craver et al, 2001).

In contrast, expression of N-cadherin de novo in breast carcinoma cells induces EMT (Hay, 1995; Hazan et al, 2000). Surprisingly, in these cellular contexts, N-cadherin behaves as a weak intercellular adhesion system. The mechanism by which N-cadherin can overcome the maintenance of the epithelial state by E-cadherin is unknown; however, a domain in N-cadherin that is essential for this effect has been identified (Khoury et al, 2005). Furthermore, the process in which up-regulation of N-cadherin upon EMT initiation occurs is also unidentified. On the other hand, the mechanisms by which these epithelial lose E-cadherin expression have been extensively described. For instance, Snail can downregulate transcription of the E-cadherin gene through its interaction with E boxes in the proximal region of the promoter (Blanco et al, 2002). Snail is expressed mostly in dedifferentiated breast tumors and is correlated with grading (Yokoyama et al, 2001). In heterogeneous breast tumors, Snail is expressed in carcinoma cell islands devoid of E-cadherin and is found in all ductal invasive carcinomas with lymph node involvement (Battle et al, 2000; Cano et al, 2000). Slug can also bind to the same region of the promoter and downregulate E-cadherin expression, although with lower affinity (Hajra et al, 1999; 2002). Other transcription factors also inhibit the transcription of E-cadherin genes: an example is the zinc finger protein SIP1, a downstream target gene in the TGF β -mediated induction of EMT in many cell lines (Xiao et al, 1999; Comijn et al, 2001).

A. Can we target E-cadherin pathway therapeutically?

Restoration of E-cadherin-mediated cell adhesion is a process that may prevent EMT in cancer. Several pathways may directly affect the adhesive properties of E-cadherin; these pathways include tyrosine kinases and tyrosine phosphatases, such as PTP-LAR (Levea et al, 2000). Blocking the transcriptional repressors such as

Snail, SIP1 and E2A (Cano et al, 2000) might also make it possible to restore E-cadherin production (Perez-Moreno et al, 2001). In this context, the Rho and Rac pathways must be further investigated because they interfere with the stability of adherens junctions. Candidate genes that encode the receptors and ligands of the ephrin and semaphorin superfamilies, (Tamagnone et al, 1999; Tamagnone and Comoglio, 2000) both of which are involved in mapping the routes of motile cells, might also be involved in the EMT of cancer cells. These proteins may represent new therapeutic targets. In studying the mechanisms that induce nuclear translocation of β -catenin, researchers may develop new targets. For example, the integrin-mediated activation of the integrin-linked kinase (ILK) has been implicated in EMT in a colon cell line. ILK inactivates glycogen synthase kinase-3, an important controller of β -catenin in the WNT pathway (Wu and Dedhar, 2001).

Finally, regarding the EMT seen in breast cancer, it might be a good idea to explore the benefits of MTA3 overexpression to restore E-cadherin expression, especially in ER-positive breast cancers (Fujita et al, 2003)

III. The receptor kinase pathway

The flow of information from the extracellular environment into the cell is at the core of a functional biological system. Receptor tyrosine kinases (RTKs) are primary mediators of many of these signals and thus determine whether the cell grows, differentiates, migrates, or dies. RTKs are cell surface allosteric enzymes consisting of a single transmembrane domain that separates an intracellular kinase domain from an extracellular ligand-binding domain. Ligand binding induces, in many instances, receptor homo- or heterodimerization, which is essential for activation of the tyrosine kinase and subsequent recruitment of target proteins. This initiates a complex signaling cascade that leads into distinct transcriptional programs (for instance, fos, jun, myc, Sp1, Egr1, as well as Ets family members) (Schaeffer et al, 1998). Many of the known tyrosine kinase receptors have been implicated in breast cancer invasion and metastasis. In many of the cases, EMT also played a role. However, more evidence is necessary to link tyrosine kinase and EMT to breast cancer.

A. ErbB receptors

The ErbB family of RTKs consists of four receptors: epidermal growth factor receptor (EGFR or ErbB1), ErbB2, ErbB3 and ErbB4. Extensive receptor-receptor interactions and the existence of a wide group of ligands underlie the enormous potential for diversification of biological messages mediated by the ErbB family. There are several ErbB-specific ligands, EGF, amphiregulin (AR) and transforming growth factor- α (TGF- α), which bind specifically to ErbB1, cellulin (BTC), heparin-binding EGF (HB-EGF) and epiregulin (EPR) (Shelly, 1998), which exhibit dual specificity for ErbB1 and ErbB4. A third group is composed of the neuregulins (NRG, also called Neu differentiation factors, NDFs, or heregulins, HRG) and includes two subgroups based on

their capacity to bind ErbB3 and ErbB4 (NRG-1 and NRG-2) or only ErbB4 (NRG-3 and NRG-4) (Harari et al, 1999). Each of the ligands has a different preference for stabilizing distinct receptor dimers; each receptor dimer has a different set of tyrosine autophosphorylation sites, which serve as a docking site for specific SH2-containing proteins and recruit different combinations of signaling molecules (Di Fiore et al, 1990; Olayioye et al, 2000).

Conversely, ErbB2 is activated only by heterodimerization with another ligand bound ErbB family member (Alimandi et al, 1995; Beerli et al, 1995). At least nine different homo- and heterodimers of ErbB proteins exist, but their formation displays a distinct hierarchy. In this network, ErbB2 plays a major coordinating role because each receptor with a specific ligand appears to prefer ErbB2 as its heterodimeric partner (Tzahar et al, 1996; Graus-Porta et al, 1997). This preference is further biased upon overexpression of ErbB2, as seen in many types of human cancer cells. ErbB2-containing heterodimers are characterized by extremely high signaling potency because ErbB2 dramatically reduces the rate of ligand dissociation. This allows strong and prolonged activation of downstream signaling pathways (Alimandi et al, 1995; Beerli et al, 1995; Graus-Porta et al, 1995; Holbro et al, 2003).

ErbB2 over expression is implicated in both breast cancer cell invasion and poor prognosis in Src- (Tan et al, 2005), mitogen activated protein kinase (MAPK), (Olayioye et al, 2000) as well as phosphatidylinositol-3 kinase (PI-3K)-dependent manner (Fedi et al, 1994; Basso et al, 2002; Nicholson et al, 2003) (**Figure 1**). Furthermore, ErbB2 cooperates with other RTKs, such as c-Met to disrupt epithelial morphogenesis and stimulate the breakdown of cell-cell junctions, dispersal and invasion of single cells (Niemann et al, 1998). Effects that are closely correlated with decrease in junctional proteins like claudin-1 and E-cadherin, in addition to the internalization of the tight junction protein ZO-1, implicate ErbB2 in the induction of invasion/metastasis through induction of EMT. Moreover, ErbB1 over expression correlates with metastasis in a variety of carcinomas, including breast (Lu et al, 2003). Finally, the expression of the mesenchymal protein, vimentin in high-grade breast tumors (grade 3) with invasiveness and chemoresistance was positively correlated with the over expression of EGFR or ErbB2 (Wade et al, 1982; Sommers et al, 1992; Korsching et al, 2005; Radovic et al, 2005). It is evident that induction in metastasis by the ErbB family members over expression or deregulation at least in part occurs through their ability to induce EMT (Matthay et al, 1993).

1. Targeting the ErbB family members

Several approaches have been utilized to target the ErbB family, but the most promising progress has been achieved in two areas: humanized antibodies against the receptor extracellular domains and small-molecule tyrosine kinase inhibitors.

i. Antibodies

In general, antibodies bind to the extracellular

domain of the receptors, inhibiting their activation by ligand and promoting receptor internalization and down regulation. At present, the most advanced of this drug type against EGFR is a chimeric antibody-IMC-C225-which is developed by ImClone Systems and Bristol-Myers Squibb (Cetuximab, Erbitux). The other important anti-receptor drug is trastuzumab (Herceptin), which was developed by Genentech. This humanized monoclonal antibody against ErbB2 has proven to be effective against breast carcinomas in which ErbB2 is highly expressed, which accounts for 20-30% of cases of metastatic breast cancer (Figure 2).

ii. Small molecules

In general, small molecules competitively inhibit ATP binding to the receptor, thereby hindering autophosphorylation and kinase activation. Such molecules are the reversible small-molecule inhibitors of EGFR, ZD1839 (gefitinib, Iressa; AstraZeneca) and OSI-774 (erlotinib, Tarceva; OSI Pharmaceuticals). Other EGFR directed small-molecule tyrosine kinase inhibitors in early stage trials include PKI116 (Novartis), GW2016 (GlaxoSmithKline), EKB-569 (Genetics Institute/ Wyeth-Ayerst) and CI-1033 (Pfizer) (Figure 2).

B. IGF receptors

IGF-IR is an evolutionary conserved, ubiquitous transmembrane tyrosine kinase, structurally similar to the insulin receptor (IR) (Ullrich et al, 1986). IGF-IR is composed of two extracellular subunits and two intracellular subunits. The subunits bind ligands (IGF-

I, IGF-II and insulin at supraphysiological doses), while subunits transmit ligand-induced signal. The subunits contain three major domains: the juxtamembrane, the tyrosine kinase and the C-terminus domains. Binding of ligands to IGF-IR induces its autophosphorylation and tyrosine phosphorylation of IGF-IR substrates, especially the IR substrate 1 (IRS-1) and Src and collagen-homology (Shc) protein. Tyrosine-phosphorylated IRS-1 and Shc bind different effector proteins (enzymes and/or adapters) inducing multiple signaling cascades, among them several interconnection pathways controlling cell survival and proliferation (Surmacz, 2000) (Figure 1).

The critical survival pathway activated by IGF-I stems from IRS-1. IRS-1 recruits and stimulates the PI-3 kinase (PI-3K), which then transmits a signal to the serine/threonine kinase Akt (Akt). Activated Akt phosphorylates and blocks a variety of proapoptotic proteins, including BAD, caspase-9, forkhead transcription factors and the GSK-3 kinase. Furthermore, Akt induces the expression of antiapoptotic proteins, such as Bcl-2 (Dews et al, 2000). Other mitogenic/survival IGF-IR pathways involve signal transducers and activators of transcription (STATs) that are phosphorylated and activated by IGF-I through JAK1/2 and PI-3K/Akt pathways (Nguyen et al, 2002; Yu et al, 2002). While antiapoptotic and growth pathways of IGF-IR have been extensively studied, the signals controlling nonmitogenic functions of IGF-IR, such as cell-substrate adhesion, migration, invasion, or intracellular interactions are less understood.

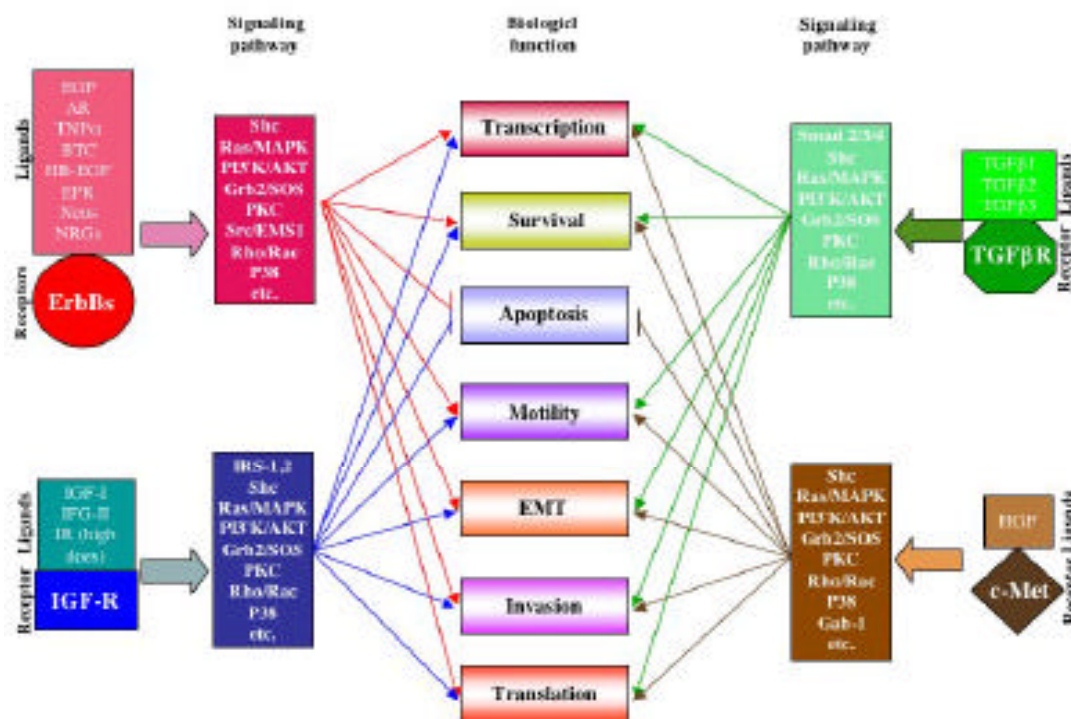


Figure 1. Schematic representation of the possible signal cascades involved in HER, c-Met, IGF, and TGF- receptors activation.

Increasing evidence demonstrates that IGF-IR pathways interconnect with integrin and cadherins signaling systems (Guvakova and Surmacz, 1999; Mauro et al, 2003; Vuori and Ruoslahti, 1994). In some experimental models, IGF-IR has been shown to mediate metastasis, possibly through enhanced migration (Doerr and Jones, 1996), reduced cell-cell adhesion (Morford et al, 1997, Valentinis et al, 1999; Mauro et al, 2003) and upregulation of plasminogen activator uPA and matrix metalloproteinases (Mira et al, 1999; Zhang and Brodt, 2003). These molecular events correlate well with EMT and thus link IGF-IR signaling with cell-cell adhesion at the molecular level. In fact, IGF-IR can regulate cell aggregation and intercellular adhesion mediated by cadherins and cadherin-associated proteins. Furthermore, IGF-IR-mediated cell-cell adhesion was blocked with an anti-E-cadherin antibody and was not observed in E-cadherin-negative MDA-MB-231 breast cancer cells (Guvakova and Surmacz 1999). Even though the available data do not suggest a firm implication of IGFRs in the EMT process leading to invasion/metastasis of breast cancer, many studies support a strong inclination that it does.

1. Targeting IGF-IR

The greatest challenge in targeting IGF-IR is designing strategies that would specifically inhibit IGF-IR without blocking IR and producing diabetogenic effects (Ullrich et al, 1986). Inhibition of either IGF-IR/ligand binding, IGF-IR expression, or IGF-I signaling can exert antitumor effects (Figure 2).

i. Antibodies

The mouse mAb -IR-3 raised against the domain of IGF-IR (Jacobs et al, 1986) inhibited IGF-IR activation and IGF-IR-dependent mitogenicity in several cell types *in vitro*, including breast carcinoma (Arteaga et al, 1992; Kalebic et al, 1994). However, in some cases -IR-3 was ineffective in blocking IGF-I-sensitive tumors in animal models (Arteaga, 1992; De Leon et al, 1992; Hailey et al, 2002). Several other mouse anti-IGF-IR mAbs were described. One of them, mAb 1H7, which blocks IGF-IR/IGF-I binding and IGF-IR-dependent DNA synthesis (Li et al, 2000; Sachdev et al, 2003) (Figure 2).

ii. Small molecule

The first described IGF-IR inhibitors, tyrphostins AG 538 and I-OMeAG, were modeled on the IR tyrosine kinase. The compounds inactivated the IGF-IR tyrosine kinase by blocking the substrate-binding site; however, cross reactivity with the IR tyrosine kinase was reported. Recent advances in the characterization of the three-dimensional structures of IGF-IR and IR greatly facilitated the design of specific IGF-IR inhibitors (De Meyts and Whittaker, 2002). Most importantly, crystallographic studies reveal conformational differences in the phosphorylated forms of IGF-IR and IR kinases, the feature allowing the development of selective therapeutics (Favelyukis et al, 2001). Several new compounds with enhanced specificity towards IGF-IR and low cross

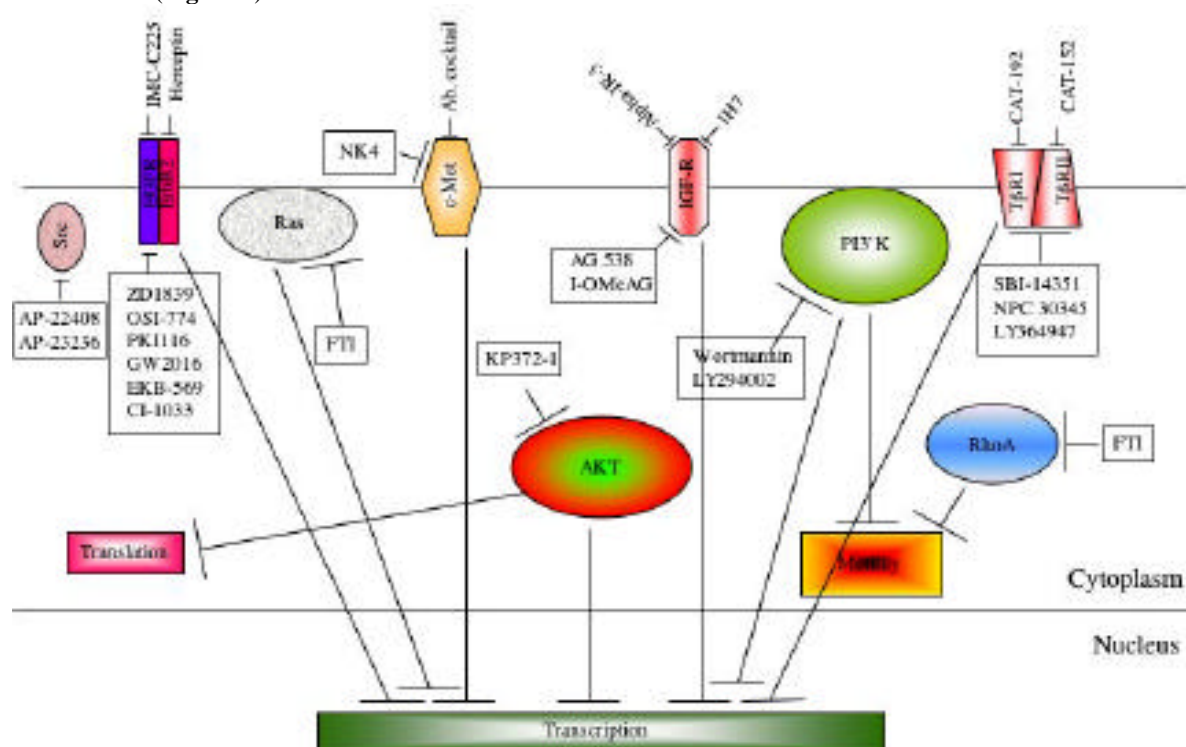


Figure 2. Strategies to inactivate ErbB, c-Met, IGF-I and TGF receptors and their down-stream signaling molecules. (a) receptors function can be blocked with inactivating Abs. Binding of Abs to receptors prevents ligand binding and induces receptor degradation. (b) Receptors tyrosine kinase activity can be abolished with small-molecule inhibitors. (c) Small molecules can also be used to inactivate Receptors down-stream signaling molecules.

reactivity with IR entered into preclinical studies. Specific small inhibitors of IGF-IR are likely to become anti-IGF-IR drugs (Pietrzkowski et al, 1992, 1993).

C. TGF receptor

The transforming growth factor (TGF) family comprises a superfamily of ligands that include the TGFs, activins and bone morphogenetic proteins (BMPs). There are three mammalian TGF isoforms, TGF 1 (Dickson et al, 1995; Shull et al, 1992), TGF 2 (Sanford et al, 1997) and TGF 3 (Kartinen et al, 1995; Proetzel et al, 1995), which, in general, exhibit similar function *in vitro* is most notable on cell growth regulation, extracellular matrix production and immune modulation (Massague, 1998; Miettinen et al, 1994). During development, TGF- 2 is a candidate inducer of EMT in the atrioventricular canal of the embryonic heart, whereas TGF- 3 is responsible for EMT following palate fusion (Kartinen et al, 1995). In EMT in the chick heart, TGF- 2 induces Slug, one of the key transcription factors in EMT (Romano et al, 2000). Similarly, mouse mammary epithelial NMuMG cells that have been made autocrine for TGF- signalling become invasive and metastatic through EMT in a p38 MAPK activation/integrin signaling-dependent fashion (Bhowmick et al, 2001b). Activation of the small GTPase RhoA, or its downstream target Rho-kinase, appeared to be more significant in this model (Bhowmick et al, 2001a) (**Figure 1**).

The TGFs bind to a heteromeric complex of transmembrane serine/threonine kinases, the type I and type II receptors (T RI and T RII) (Wrana et al, 1994). Following ligand binding to T RII, T RI is recruited to ligand receptor complex, allowing the constitutive activation of T RII kinase, which in turn transphosphorylate and activate the T RI kinase (Wrana et al, 1994). Activated T RI phosphorylates the receptor-regulated Smad2 and Smad3. Finally, Smad2 and Smad3 then associate with the common mediator Smad4 and move to the nucleus where they regulate gene transcription (Massague et al, 2000). By contrast, the inhibitory Smad7 can interact with T RI and prevent the phosphorylation of effector Smads (Hayashi et al, 1997). In addition to Smads, other signaling pathways have been implicated in TGF actions. These include the Erk, JNK and p38, PI3K and Rho GTPases (Derynck et al, 2001; Wakefield et al, 2002). The roles of these non-Smad pathways in mediating the cellular effects of TGF remain to be fully characterized. Several reports support a causal association between an excess of endogenous or exogenous TGF and breast tumor progression (Arteaga et al, 1993; Siegel et al, 2003). There is also evidence that high production and/or activation of TGF in tumors can enhance cancer progression by autocrine and/or paracrine mechanisms (Dumont and Arteaga, 2000; Derynck et al, 2001; Wakefield et al, 2002) (**Figure 1**).

Evidences support the idea that TGF induces EMT in tumor and in non-tumor epithelial cells (Miettinen et al, 1994; Oft et al, 1996). For example, expression of T RII in colon cancer cells (with low invasive potential) restores tumor cell invasiveness (Oft et al, 1996). Forced

expression of dominant-active Smad2 in squamous cancer cells result in enhanced tumor cell motility and metastasis dissemination (Oft et al, 2002). This evidence further supports that the tumor-promoting role of autocrine TGF, expression of dominant-negative T RII in metastasis cancer cells, prevents EMT while inhibiting motility, tumorigenicity and metastases (Dumont and Arteaga, 2003). This data suggests that TGF may select for more metastatic cancers (Cui et al, 1996). Recently, over expression of active TGF 1 or activated T RI in the mammary gland of transgenic mice has been shown to accelerate metastases derived from neu-induced primary mammary tumors (Watanabe et al, 2001; Siegel et al, 2003), suggesting that loss of autocrine TGF signaling may limit systemic metastases.

TGF-family members cooperate with either RTKs or their downstream signal transducers (Iascone et al, 1999). This cooperation can overcome the well-known tumor suppressive effects of TGF signaling (cell cycle arrest, apoptosis induction, Herzer et al, 2005). The same cooperation also allows TGF signaling to modulate epithelial plasticity and migration/motility, a process crucial for tumor progression and metastasis (Blanco et al, 2002). Importantly, TGF R and RTK signaling converge at the level of transcriptional regulation (Janda et al, 2002; Siegel et al, 2003). This cooperation may involve separate activation of different transcription factors with similar or opposing actions. These factors may regulate cell cycle progression (cdk inhibitors, D cyclins), apoptosis (pro- vs. antiapoptotic proteins), migration (Net1, Rho-A, PI3K, ERK/MAPK) and epithelial adhesion/plasticity (E-cadherin repressors, snail, EF-1, EMT-genes and genes of the -catenin signaling pathway) (Eger et al, 2000; Hemavathy et al, 2000; Keller et al, 1999; Reichmann et al, 1992).

1. Targeting TGF R signaling

The improved outcome of patients who bear cancers with T RII mutations supports an argument in favor of blocking autocrine TGF which includes a therapeutic intent (Anbazhagan et al, 1999; Tian et al, 2004). An additional rationale can be inferred from the paracrine effects of tumor TGFs on angiogenesis, stromal formation and remodeling and on immunosuppression. These observations suggest that by blocking TGF function, one can interrupt multiple events that are necessary for tumor maintenance. Indeed, preclinical studies support the principle that inhibition of TGF affects these tumor-permissive autocrine and paracrine mechanisms (Dumont et al, 2003) (**Figure 2**).

i. Antibodies

Blocking ligand access to TGF receptors using mAb is one way to effectively disrupt this signaling pathway. Two humanized monoclonal antibodies: CAT-192, specific to TGF 1 and CAT-152, against TGF 2, are in early clinical development. The expression of multiple TGF isoforms in tumors suggest that a pan-TGF antibody might be more effective than isoform-specific antibodies. Two pan-TGF monoclonal antibodies, 1D11

and 2G7, have been reported. The 2G7 pan-TGF neutralizing IgG2 suppresses the establishment of MDA-MB231 tumors and lung metastasis in athymic mice and prevents the inhibition of host natural killer cell function induced by tumor inoculation. The antibody produced no effect against MDA-MB231 cells *in vitro*, nor did it exhibit an antitumor effect in natural killer-deficient mice. This suggests that antibody mediated TGF blockade is effective in disrupting tumor-host immunosuppressive interactions that are essential for tumor establishment and metastatic progression (**Figure 2**).

ii. Small molecules

A second group of strategies is aimed at directly blocking a receptor's catalytic activity. SBI-14352, NPC 30345 and LY364947 are ATP competitive inhibitors of the T_{RI} kinase. This approach spares the T_{RII} kinase and, therefore, may not inhibit TGF function completely (Wojtowicz-Praga 2003). If complete inhibition of TGF was required for antitumor action, this selectivity could compromise anticancer activity while at the same time improve potential toxicities. These two possibilities are strictly theoretical because there are no known T_{RII} functions that do not require ThRI. Nonetheless, the development of bifunctional T_R kinase inhibitors would perhaps resolve these questions (**Figure 2**).

D. c-Met receptor signaling

Met, which was discovered as an oncogene two decades ago (Zhang and Vande Woude 2003; Gao and Vande Woude 2005), encodes for a disulphide-linked heterodimer RTK that binds to and is activated by the growth and motility factor HGF (aka, scatter factor 1), (Comoglio and Boccaccio, 2001; Zhang and Vande Woude, 2003). Phosphorylation of the S985 located in the intracellular portion of the receptor by PCK or Ca²⁺/calmodulindependent kinases (Danilkovitch-Miagkova and Zbar 2002) has an inhibitory function. Phosphorylation on the tyrosine residue (Y1003) allows binding to the E3-ubiquitin ligase Cbl, which promotes receptor ubiquitination, endocytosis and degradation (Zhang and Vande Woude 2003). However, the receptor C-terminal tail is a unique docking site for a wide spectrum of downstream signaling molecules. These molecules include PI3'K, the GRB2-SOS complex, the Src, the transcription factor signal transducer and activator of transcription 3 (STAT3) and the adaptors Shc and Gab1, which provide additional docking sites for many signaling molecules (Longati et al, 2001; Zhang and Vande Woude 2003) (**Figure 1**).

In both *in vitro* and *in vivo*, Met activation evokes pleiotropic biological responses often referred to as "invasive growth" (Nakamura et al, 1989; Naldini et al, 1991). *In vivo*, Met is expressed on epithelial cells of many organs (Kamalati et al, 1999), both during embryogenesis and in adulthood; its function is essential for embryo development (knockout mice for either Met or Hgf are embryonic ally lethal; Birchmeier and Gherardi 1998). Under physiological conditions, Met contributes to the establishment of normal tissue patterns and the onset

and maintenance of normal organ architecture such as muscle development, nervous system formation, hematopoietic differentiation, bone remodeling and angiogenesis (Birchmeier and Gherardi 1999). Moreover, Met activation plays a crucial role in the EMT that takes place during acute injury repair (Otonkoski et al, 1996; Yu and Merlino, 2002; Lengyel et al, 2005). Moreover, HGF-activated Met receptor stimulates tyrosine phosphorylation of FAK and induces actin reorganization during migration (Weidner et al, 1993; Comoglio and Boccaccio, 2001; Zhang and Vande Woude, 2003; Wasenius et al, 2005).

Met cooperates with different cell surface molecules. For example, c-Met associates with CD44, which is a major component of the extra cellular matrix. It also correlates well as integrin $\alpha 6 \beta 4$, where the activity of the integrin is independent from its adhesive role because it forms an additional signaling platform necessary for the complete promotion of Met-induced invasive growth (Lengyel et al, 2005). Met also associates with all three members of class B plexins (transmembrane receptors for semaphorins), (Comoglio et al, 2002; Conrotto et al, 2004). These interactions have functional roles; over expression or stimulation of B plexins by their ligands induces scatter factor receptor activation and promotes the invasive-growth program (Giordano et al, 2002). Furthermore, c-Met interacts with other surface RTKs, physically in the case of EGF receptor (Jafri et al, 2003), or through synergism with intercellular signaling in the case of ErbB2 in promoting a malignant phenotype (Muller and Park, 2005; Saucier et al, 2004; Swiercz et al, 2004).

Until recently, RTK activity was believed to be modulated in different tissues on the basis of ligand availability, expression levels of the receptors and in the presence of a different panel of intracellular transducers. With these cross-talk interactions, the activity of some receptors such as Met could depend on the simultaneous expression and/or activation of other membrane receptors. Thus unveiling a new possibility for RTK control. Activated c-Met recruits several SH2-domain-containing proteins, including adaptor proteins (such as Grb2, Shc, Gab1 and Cbl) and effector proteins (such as phosphatidylinositol 3- kinase (PI3'K), the tyrosine kinase Src, phospholipase C (PLC), the protein tyrosine phosphatase Shp2 and the transcription factor signal transducer and activator of transcription 3 (Stat3, Laird et al, 2003). Gab1, which amplifies the Met response, stimulates branching morphogenesis *in vitro* by activating Shp2 and PLC in a sustained manner (Fixman et al, 1996).

1. Targeting c-Met signaling

Approaches to block ligand-dependent Met activation have been developed.

i. Antibodies

A process to neutralize anti-HGF antibodies exists; however, it has been shown that, with available reagents, a minimum of three antibodies (each one with its own pharmacodynamic features) against different HGF epitopes are required to completely inhibit Met activation

(Jiang et al, 2005). Unless new antibodies are developed, these results raise concerns about the feasibility of this approach (Figure 2).

ii. Small molecules

Alternatively, strategies that directly target the receptor can block both HGF-dependent and HGF-independent Met activation. The inhibition of Met kinase activity has been achieved through small ATP competitors (Sawyer et al, 2004). The molecules present a problem as a result of their selectivity. The available Met inhibitors are not specific; possible side effects raise concern for patients. Another approach is to interfere with HGF binding. The most thoroughly characterized HGF competitor is NK4 (Heideman et al, 2004), a molecule composed of the N-terminal hairpin and the four-kringle domain of HGF. NK4 binds to Met without inducing receptor activation and thus behaves as a full antagonist (Heideman et al, 2004) (Figure 2).

IV. The cytoplasmic signal transduction pathway

EMT can also be induced *in vitro* in several epithelial cell lines by over-activation of cytoplasmic signal transduction pathways, e.g., Ras/mitogen-activated protein kinase (MAPK), PI3`K, Src and Rho/Rac all have an effect on particular aspects of EMT.

A. Ras/MAPK signal transduction

Evidence suggests that Ras plays an essential role in the induction and maintenance of EMT during breast cancer progression (Rodenhuis, 1992). By using specific inhibitors and effector-specific Ras mutants, several research groups were able to show that hyperactive Raf/mitogen-activated protein kinase (MAPK) is required for EMT (Oft et al, 1996; 2002; Janda et al, 2002; Xie et al, 2004).

1. Targeting Ras signaling

The attachment of the farnesyl isoprenoid group to the H-Ras, K-Ras and N-Ras proteins is essential for the biological activity of Ras. Therefore, the design of new rational therapies against the Ras pathway is in development. A large number of highly effective farnesyltransferase inhibitors (FTIs) have been identified (Cesario et al, 2005; Frassanito et al, 2005). These were shown to efficiently inhibit the farnesylation of H-Ras in cells in culture, which led to high expectations of being effective against the 20% of human tumors that have activating mutations in Ras genes (Frassanito et al, 2005). Unfortunately, this early potential has not been realized. The mode of action of FTIs has become increasingly unclear and the initial spectacular successes that were achieved in mouse models have not been reported in human patients. Despite uncertainty about their mechanism of function, FTIs do have marked effects on the growth and survival of some tumor cell lines *in vitro* and on xenografts in nude mice, although not necessarily those expressing activated Ras. The effects of FTIs in these pre-clinical systems have been reviewed extensively

(Hahn et al, 2001; Baum and Kirschmeier, 2003) (Figure 2).

B. PI3`K/AKT signal transduction

Phosphatidylinositol-3 kinases, PI3`Ks constitute a lipid kinase family characterized by their ability to phosphorylate inositol ring 3-OH group in inositol phospholipids to generate the second messenger phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P3) (Carpenter and Cantley, 1996). RTK activation results in PI(3,4,5)P3 and PI(3,4)P2 production by PI3`K at the inner side of the plasma membrane. Akt interacts with these phospholipids, causing its translocation to the inner membrane, where it is phosphorylated and activated by PDK1 and PDK2 (Anderson et al, 1998; Toker and Newton, 2000). Activated Akt modulates the function of numerous substrates involved in the regulation of cell survival, cell cycle progression and cellular growth. In recent years, it has been shown that PI3`K/Akt signaling pathway components are frequently altered in human cancers. For example, amplification and activation mutations have been detected in the PI3`K gene. The gene encodes the p110 catalytic subunit of PI3`K and is located in the chromosome 3q26, a region that is frequently amplified in several human cancers, including breast, ovarian and cervix cancer. No modifications or mutations in the akt gene have been found in mammals. Nonetheless, various studies have found akt amplifications in human cancers, such as Akt2 gene amplifications in ovarian, pancreas, breast and stomach tumors (Bellacosa et al, 1995; Ruggeri et al, 1998; Chau and Ashcroft, 2004).

Survival signals induced by several receptors are mediated mainly by PI3`K/Akt, hence this pathway may play a major role in drug resistance appearance. In fact, there is convincing evidence from recent research suggesting that PI3`K/Akt pathway activation is related to tumor cell resistance to both chemotherapy and radiation. Moreover, it has been suggested that activation of Akt1 by ErbB2/PI3`K plays an important role in mediating multidrug resistance in human breast cancer cells (Mills et al, 2003) and that Akt may therefore be a novel molecular target for therapies that would improve the outcome of patients with breast cancer (Kelland, 2005). In ovarian cancer, aberrant Akt expression or activation in different cell lines has been able to confer paclitaxel resistance (VanderWeele et al, 2004). It has also been reported that PI3`K inhibition increases paclitaxel efficiency in *in vivo* and *in vitro* ovarian cancer models (Hu et al, 2002). Moreover, it has been shown that integrin-mediated protection to paclitaxel- and vincristine-induced apoptosis is dependant on PI3`K/Akt signaling pathway activation (Aoudjit and Vuori, 2001).

1. Targeting PI3`K/Akt signaling pathway

Wortmannin is a fungal metabolite and a potent inhibitor of type I PI3`K. Wortmannin has antitumor activity *in vitro* and *in vivo* studies, with an IC50 range for inhibition of PI3`K from 2 to 4 nM (Wymann et al, 1996; Mills et al, 2003). Based upon the potent inhibitory effect of wortmannin *in vitro* assays, additional studies in animal models have been conducted to test the efficacy of wortmannin in inhibiting tumor growth *in vivo* (Davol et

al, 1999). Although these studies suggest that blocking the PI3'K/Akt pathway with wortmannin might be a valuable approach to treat cancer, one disadvantage of the use of wortmannin is its instability in an aqueous environment. Wortmannin is soluble in organic solvents, which may limit its use in clinical trials. Currently, water-soluble wortmannin conjugates are being developed to circumvent this issue (Okaichi et al, 2002).

The flavonoid derivative, LY294002, is a competitive and reversible inhibitor of the ATP binding site of PI3'K. Several *in vitro* studies have shown that LY294002 alone has antiproliferative and proapoptotic activities (Uddin et al, 2005). Relatively, few *in vivo* studies have been conducted to demonstrate the efficacy of LY294002 on the inhibition of tumor growth, but these studies show that the administration of LY294002 in human cancer xenografts inhibit tumor growth and induced apoptosis (Semba et al, 2002). Similar to wortmannin, the combination of LY294002 with various cytotoxic drugs or radiation enhances the effectiveness of these treatments and highlights the therapeutic potential of targeting this pathway (Semba et al, 2002). Furthermore, an AKT inhibitor such as KP372-1 was found to suppress AKT activity and cell proliferation and induce apoptosis in thyroid cancer cells (Mandal et al, 2005) (**Figure 2**).

C. Src signal transduction

The viral src gene encoded by Rous sarcoma virus (RSV) was the first defined oncogene and encodes the first recognized tyrosine kinase, v-Src. Its cellular counterpart is c-Src (Hunter and Sefton, 1980; Martin, 2001; Boyer et al, 2000). Cells that are transformed by RSV lack bundled actin filaments and a reduction in the number and size of cell-substrate adhesions (focal adhesions) into which actin filaments are tethered. This results in conversion from a well-spread morphology to a more refractile, elongated cell shape reminiscent of EMT. Furthermore, gain-of-function mutation in the v-Src SH3 domain stabilizes a complex of Src and FAK and its localization to integrin-associated invadopodia (Kellie et al, 1986; Brunton et al, 2001). This, in turn, depends on the effects of some small GTPases on cytoskeletal modeling. For example, RhoA targets Src to focal adhesions, Rac1 targets it to focal complexes along lamellipodia and Cdc42 targets it to focal complexes along filopodia (Fincham et al, 1996).

V-Src and c-Src also target cortactin, an F-actin bundling protein that localizes to podosomes and lamellipodia (Wojakowski et al, 1993; Hiura et al, 1995; Weed et al, 1998, 2000; Boyer et al, 2001; Weaver et al, 2001). Cortactin associates with and activates, Arp2/3, a protein complex that is required to nucleate the formation of actin-filament networks. This interaction occurs through an amino-terminal acidic region of cortactin that has analogous functions to similar regions in the Wiskott Aldrich Syndrome protein (WASP) family (Mizutani et al, 2002). Specifically, cortactin functions to stabilize Arp2/3-induced actin filament assembly at the cell periphery and may play a role in podosome formation (Weed et al, 2000). These observations indicate that actin regulators have a crucial function in mediating the assembly of dynamically regulated podosomes that are induced by v-

Src and that confer a migratory and invasive phenotype.

Compelling evidence that endogenous (Src family kinases (SFKs)) play a significant role in cell migration is provided by the impaired integrin-dependent migration of cells derived from mouse embryos that lack c-Src, Fyn and Yes (Matsuyoshi et al, 1992; Klinghoffer et al, 1999). In addition, loss of c-Src results in the strengthening of links between integrins and the force-generating cytoskeleton, which indicates that the normal role of c-Src or FAK is to weaken, or disrupt, such links (Ilic et al, 1995; Fincham et al, 2000).

1. Targeting Src pathway

A novel Src homology (SH)-2 inhibitors incorporating non-hydrolyzable phosphotyrosine mimics (AP-22408, Ariad Pharmaceuticals) has been evaluated (Shakespeare et al, 2003). Another approach is the ATP-based Src kinase inhibitors (AP-23236, Ariad Pharmaceuticals, Shakespeare et al, 2003). The two compounds differ mechanistically by virtue of blocking Src-dependent non-catalytic or catalytic activities in osteoclasts. This process provides the framework for the next-generation molecules that have further advanced, in terms of preclinical studies, for the treatment of osteoporosis and related bone diseases, including osteolytic bone metastases (**Figure 2**).

D. Rho and Rac family

The importance of the Rho-GTPases in cancer progression, particularly in the area of breast cancer metastasis, is becoming increasingly evident. All aspects of cellular motility and invasion, including cellular polarity, cytoskeletal organization and transduction of signals from the outside environment are controlled through interplays between the Rho-GTPases (Itoh et al, 1999; Price et al, 2001; Ridley et al, 2001; Etienne-Manneville and Hall, 2002). Rho family consists of RhoA, B and C and their homologs Cdc42, Rac1 and 2 (Nobes and Hall, 1995). Like Ras, Rho proteins are localized to the inner plasma membrane by a C-terminal lipid modification (Hall, 1990; 1998) and are able to bind GDP/GTP and hydrolyze GTP and lead to activation of downstream effector molecules, which will lead to a cellular response (Kjoller and Hall 1999; Mareel and Leroy, 2003). Interestingly, some Rho family members such as Rnd and RhoH appear to lack intrinsic GTPase activity (Dallery-Prudhomme et al, 1997; Nobes et al, 1998). Ras constitutes 5% of human breast tumors and carries an identifiable Ras mutation, which renders the GTPase incapable of hydrolyzing bound GTP, thus remaining constitutively active (Rochlitz et al, 1989). No mutation in any of the Rho proteins has been identified in human tumors. Rather, over expression of Rho proteins, particularly RhoA and RhoC, appears to be the rule in human cancers (Moscow et al, 1994; Fritz et al, 1999; Imamura et al, 1999; Clark et al, 2000; van Golen, 2000).

Rac1 forms the leading lamellipodial edge of the cell (Evers et al, 2000a and b; Mareel and Leroy, 2003). Furthermore, in cancers with weakened adherens junctions through for EGF or HGF initiated pathways, Rac is required to promote cell migration and invasion (Ridley et

al, 1995; Lamorte et al, 2002). Cdc42 forms the "ruffles" or "microspikes" known as filopodia, which redistribute the cell membranes to lamellipodium extension as the cell migrates and RhoA redistributes the actin stress fibers contracting the cell body in the direction of cell movement (Hall, 1990; 1998; Ridley 1994; Cussac et al, 1996; Price and Collard, 2001; Evers et al, 2000a and b). The ability to grow under anchorage-independent conditions signals by Rho family is through the PI3`K pathway. Motility and invasion signal through the Erk, JNK/SAPK and p38 MAPK pathways (Bouzahzah et al, 2001; Ridley, 2001; Rihet et al, 2001; Jo et al, 2002) and the production of angiogenic factors signals through p38.

Although Rho-kinase (ROCK) has been suggested to be a downstream target for both RhoA and RhoC, treatment of the cells with the pharmacological ROCK inhibitor, Y-27632, does not affect the RhoC-induced phenotype. Elucidating the mechanisms that result in Rho-over expression and activation are key in understanding the role of these proteins in breast cancer progression and metastasis. In normal cells, a fine balance maintains equilibrium between Rho GTPases in active and inactive states. Thus, perturbation of any of the Rho regulatory proteins, either through mutation, growth factor receptor dysregulation or oncogene expression can lead to aberrant Rho activation, increased motility, invasion and possibly metastasis (Kheradmand et al, 1998; Cho et al, 2000; Ozanne et al, 2000; Zhuge et al, 2001; Soon et al, 2003). With the large number of RhoGDIs, RhoGEFs and RhoGAPs thus far identified and more being continuously added to the list, the main challenge will

E. Targeting Rho GTPase pathways

Several drugs that block or decrease signaling by the Rho GTPases have now been shown to alter breast cancer morphology, cell growth and/or apoptosis. FTIs, originally designed to inhibit Ras lipid modification, also modify Rho proteins (for review, see Prendergast, 2001, Cesario et al, 2005) including RhoB. FTI treatment increases geranylgeranylated RhoB, which induces apoptosis selectively in cancer cells (Du and Prendergast, 1999). Other promising approaches involve selective inhibition of certain signaling pathways downstream of Rho GTPases. A specific PAK inhibitor decreased growth of Ras transformed cells (Nheu et al, 2002), but has yet to be tested in breast cancer cells. The ROCK effector kinase blocked metastasis of Hepatoma and may also prove effective in breast cancer cells (Itoh et al, 1999) (**Figure 2**).

V. Implications and future directions

EMT and metastases (the pathological *in vivo* correlate of EMT) are complex developmental processes that involve major reprogramming of gene expression that lead to alterations in cell fate and behavior. Many external signals induce this reprogramming through a complex signaling network; this network involves many autocrine and/or paracrine growth factor loops such as TGF or PDGF. Also, several intracellular signaling pathways such as Smads, ERK/MAPK, -catenin and PI3`K, work within this process. Within this signaling network, EMT is

regulated through three factors: signal integration, crosstalk and feedback control. However, death receptor, integrin, hedgehog, or Notch signaling pathways may at least in some cases, contribute to EMT. Therefore, during tumor progression, this complex signaling network is eventually dysregulated.

Reprogramming of gene expression towards mesenchymal traits may induce growth factor secretion and upregulate their receptors. This could cause hyperactivation of intracellular signaling, which may contribute to EMT, local invasion and metastasis. It is unclear whether EMT occurs in metastatic carcinomas. A deeper understanding of EMT will lead to the development of better therapeutic approaches for patients. Despite recent advances, researchers do not know how relevant or how frequent EMT and its induction is in human tumors. Several genetic mouse tumor models support EMT as a general mechanism in metastasis. An important aim for future research is to study the stages of EMT, both in embryos and in mouse models of carcinogenesis. In the future, candidate genes will be assessed for their contribution to EMT in human tumors by, for example, examining their function in tumors transplanted in immunodeficient mice or in transgenic mouse models. The main challenge is to discover how growth factors, scatter factors and ECM components cooperate to induce EMT. An extensive understanding of the processes that trigger EMT will lead researchers to develop ways to prevent it. This therapeutic strategy has the potential to block metastases. This could possibly prevent cancer recurrence because micrometastases often remains after conventional surgery, radiotherapy and/or chemotherapy.

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