Overexpression of EGFR and c-erbB2 causes enhanced cell migration in human breast cancer cells and NIH3T3 fibroblasts

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Abstract Overexpression of EGFR and c-erbB2 frequently occurs in human breast cancers, correlating with poor prognosis. Here we show that overexpression of EGFR and c-erbB2 in cell lines increases cell migration, an important step in metastasis formation. The effect of EGFR on migration is dependent on the addition of EGF to the cells. In contrast, c-erbB2 seems to act independently of its ligand in these assays. Overexpression of this receptor is sufficient to induce cell migration. In addition, we investigated the involvement of a number of signal transduction pathways known to be activated by the EGFR. We found that inactivation of MAPKK results in a decreased migration, while inactivation of PI3K increases migration.

Key words: Epidermal growth factor receptor; c-erbB2; Overexpression; Cell migration; Human breast cancer

1. Introduction

The epidermal growth factor receptor (EGFR) and c-erbB2 are members of the type 1 family of tyrosine kinase cell surface receptors. Activation of the EGFR can lead to proliferation or differentiation depending on the cell type [1]. Upon ligand binding, the EGFR can homodimerize, or form heterodimers with the other members of the type I family [2]. Receptor dimerization leads to activation of the EGFR and subsequently to cross-phosphorylation of tyrosine residues in the cytoplasmic tail of the receptor [1]. These phosphotyrosine residues in the carboxy-terminus of the receptor serve as high affinity sites for proteins that, in turn, transmit the growth factor signal inside the cell [3]. Examples are phospholipase C-γ [4], the 85 kDa subunit of phosphatidylinositol-3 kinase [5], c-Src tyrosine kinases [6], and Grb2 [7]. Although the possible ligand for c-erbB2 has not been identified yet, the C-terminus of the receptor can be phosphorylated by cross-phosphorylation through EGFR. Several reports have described the purification of a biologically active ligand, termed heregulin or neu differentiation factor. However, direct binding of this factor to c-erbB2 has not been demonstrated (reviewed in [8]).

Overexpression of the EGFR and also c-erbB2 has been found in a number of human malignancies, including bladder cancer [9], colon carcinoma [10], and lung cancer [11]. In breast cancer, high levels of the EGFR [12] and c-erbB2 [13] have been shown to correlate strongly with poor prognosis. An apparently additive effect has been described of EGFR and c-erbB2 overexpression on patient prognosis [13,14].

A common mechanism for c-erbB2 overexpression in breast cancer is gene amplification, but increased c-erbB2 protein is also found in some cancers with normal gene copy number [13]. It has been shown that overexpression of c-erbB2 alone is sufficient to cause malignant transformation [15]. Unlike c-erbB2, however, EGFR overexpression is almost never due to gene amplification, rather increased receptor synthesis appears to be responsible [16]. EGFR, even when significantly overexpressed, does not transform unless EGF or TGF-α are provided [17]. The expression of EGF, TGF-α, and another EGF family member, named amphiregulin, is often increased in breast cancers [18].

The final stage of tumor progression is formation of metastases. Metastatic spread of tumors is a consequence of a series of events in which growth factors could be involved. Sequentially, tumor cells must proliferate, lose their contacts with neighboring cells, pass through the vessel wall, enter the blood stream, infiltrate the target organ and form a secondary tumor [19]. During all these processes, tumor cells are subjected to a variety of environmental controls, including growth factors from the host or from the tumor itself. Accumulating evidence suggests that activation of the EGFR by EGF or TGF-α may support the metastatic capacity of tumor cells.

In the present report we studied the effects of EGFR and c-erbB2 overexpression and activation on cell migration, an important step in the metastatic process. We used human breast cancer cells expressing EGFR and NIH3T3 cells expressing EGFR, EGFR/c-erbB2 chimerae, or c-erbB2. The results show that cell migration of human breast cancer cells that overexpress EGFR is highly (20-fold) increased upon EGF incubation. Using inhibitors for MAPKK and PI3K, we found that both MAPK and PI3K pathways might be involved in this phenomenon.

In NIH3T3 cells overexpressing EGFR or c-erbB2, cell migration is also increased. Our results indicate that cell migration of EGFR overexpressing cells is only increased after EGF incubation, whereas overexpression of c-erbB2 alone is sufficient to induce cell migration.

These results show that overexpression of both EGFR and c-erbB2 induces in vitro cell migration, a process that is involved in the metastatic spread of tumor cells.

2. Material and methods

2.1. Cell culture

ZR75-1 human breast cancer cells, transfected with the EGF recep-

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tor (ZR11) or the empty vector (ZR9b11) [20], were kindly donated by E. Valverius (Uppsala, Sweden). HER13 and HER14 cells (NIH3T3 cells transfected with wild type EGFR [21]) were kindly donated by J. Boonstra (Utrecht, The Netherlands). LTR-EN2 and NEN37 cells (NIH3T3 cells transfected with a EGFR/c-erbB2 chimera [22]) were kindly donated by K. Alitalo (Helsinki, Finland). H3 and 3.4 cells (NIH3T3 cells expressing c-erbB2 under the control of a tetracycline-responsive promoter and cells expressing c-erbB2 from a constitutive SV40 early promoter respectively [23]) were kindly donated by T. Beckers (Asta Medica AG, Frankfurt/Main, Germany). NIH3T3 cells were obtained from the American Tissue Culture Collection.

Cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated newborn calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, in a 5% CO_2 humidified atmosphere at 37°C.

All cell lines expressed the respective receptor or chimera, which could be phosphorylated, as was confirmed by immunoprecipitation of the receptor/chimera followed by Western blotting experiments using anti-EGFR or anti-c-erbB2 antibodies or using anti-PTyr antibodies (data not shown).

2.2. Cell migration assay

We used Transwell cell culture chambers (Costar 3422, Cambridge, MA) containing filters with 12 μm pore size. The filters were either left untreated or coated with extracellular matrix (70 μl of a 1:7.5 dilution in H_2O; ECM is a reconstituted basement membrane extract from the EHS (Engelbreth-Holm-Swarm) sarcoma, containing laminin, collagen type IV, and heparin sulfate proteoglycan, Harbor Bio-products, Norwood, MA). At the bottom of the lower compartment different concentrations of EGF dissolved in 100 μl low-melting agarose were placed as a chemoattractant. The EGF/agarose solution was added along the sides of the lower compartment and 1500 μl culture medium was added. Cell suspensions (5×10^4 cells/500 μl culture medium) were added to the upper compartment. After 48 h the number of migrated cells at the bottom of the lower compartment was counted under a phase-contrast microscope in 10 random fields at a 100× magnification. The assays were performed in triplicate and repeated three times.

3. Results

In order to study the effect of EGFR and c-erbB2 overexpression on the metastatic process, we developed an in vitro cell migration system. In vivo, tumor cells have to overcome barriers, like the vessel wall, in order to form a secondary tumor. To mimic this situation we used Transwell cell culture chambers, that contain filters with 12 μm pore size. We coated these filters with extracellular matrix (ECM) components. Since activation of EGFR by its ligand EGF has been described to induce metastatic spread of tumor cells, we studied the induction of cell migration by EGF. Therefore, we placed EGF, dissolved in low-melting agarose, as a chemoattractant at the bottom of the lower chamber (see Material and Methods). This approach worked far better than just adding EGF in culture medium in the lower compartment, since much more cells migrated when EGF was first dissolved in agarose (not shown).

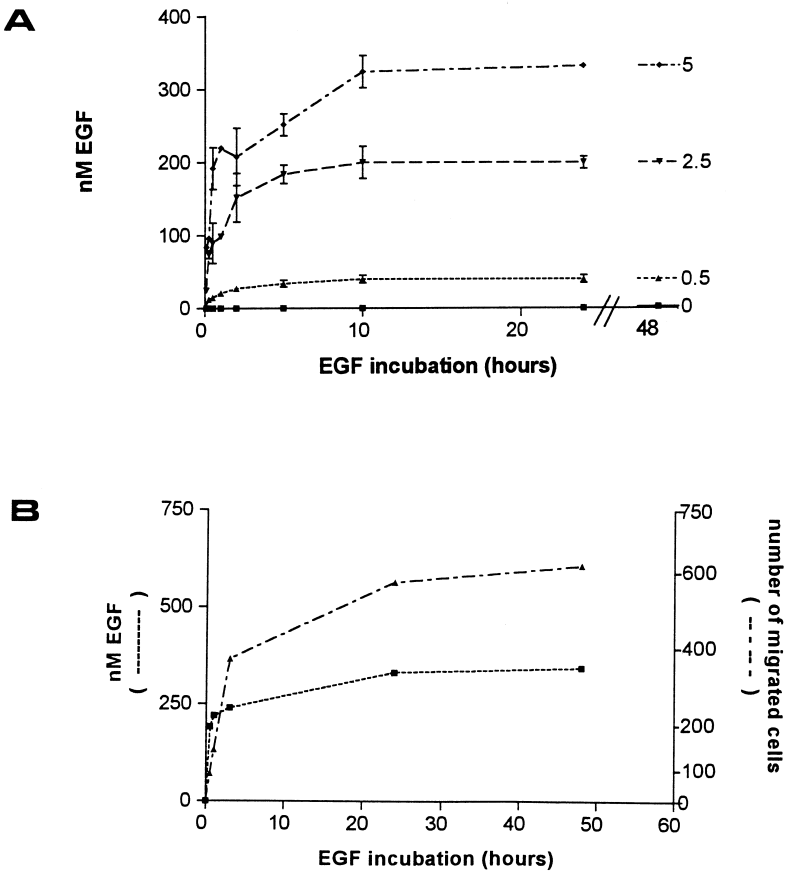

Fig. 1. In vitro assay for cell migration. A: 0, 0.5, 2.5 and 5 ng ^{125}I-EGF was dissolved in 100 μl low-melting agarose and placed at the bottom of the underwell of Transwell cell culture chambers. At several time points samples were taken from the upper well and radioactivity was determined. The EGF concentration (nM) was calculated from the radioactivity released from the agarose, using a standard curve. B: ZR11 cells were incubated with 5 ng EGF/100 μl agarose. At various time points the number of migrated cells was counted under a phase-contrast microscope in 10 random fields (100× magnification). Corrections were made for proliferation of the migrated cells. EGF concentration (nM) at various time points is shown.

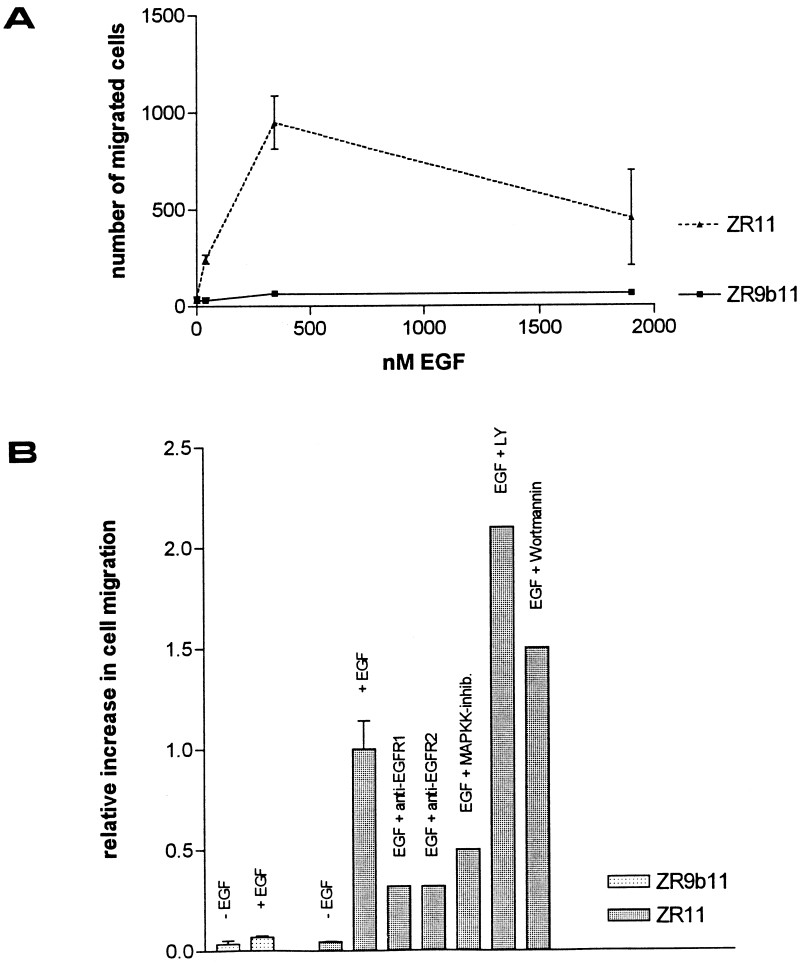

Fig. 2. Cell migration in EGFR overexpressing human breast cancer cells. A: ZR9b11 and ZR11 cells were incubated with 0, 0.5, 5, or 25 ng EGF/100 μl agarose. After 48 h the number of migrated cells was counted under a phase-contrast microscope in 10 random fields (100× magnification). Representative experiment out of three, performed in triplicate (mean ± S.D.). B: ZR9b11 and ZR11 cells were incubated with 0 or 5 ng EGF/100 μl agarose. In addition, ZR11 cells were incubated with 5 ng EGF/100 μl and other factors, as indicated. After 48 h the number of migrated cells was counted. Relative cell migration is shown, compared with the number of migrated ZR11 cells after 48 h incubation with 5 ng EGF/100 μl.

To study the kinetics of EGF release from the agarose, we added ^{125}I-labelled EGF to the agarose and took samples from the upper chamber at different time points to measure radioactivity. We used a standard curve to correlate the radioactivity to the ^{125}I-EGF concentration that resulted from the ^{125}I-EGF release from the agarose. Only after 10 hours most of the EGF was released (Fig. 1A), suggesting a gradual EGF release up to 10 hours after EGF addition.

Next, we investigated whether cell migration in this system was also a gradual event. Therefore we used human breast cancer cells (ZR11, transfected with EGFR), since EGFR overexpression is often found in this type of cancer. We determined the number of migrated ZR11 cells after different time intervals of EGF incubation (5 ng/100 μl agarose, maximum molarity 400 nM EGF) in the presence of 10% serum. Fig. 1B shows that the number of migrated ZR11 cells increases at least until 48 h after addition of the cells to the upper chamber. The increase in migrated cells parallels the increase in EGF concentration, suggesting that EGF is responsible for this migration.

Furthermore, to mimic the situation in vivo we decided to incubate the cells with EGF in the presence of 10% serum. Proliferation of cells growing on 10% serum was the same as for cells growing on 10% serum and additional EGF (up to 50 ng/ml, not shown). In our assays corrections are made for proliferation of the migrated cells by dividing the cell numbers by a factor determined in parallel proliferation experiments.

In conclusion, Fig. 1 shows that EGF is gradually released from the agarose, resulting in an increasing EGF concentration in the culture chamber. The number of migrated cells also gradually increases up to 48 h after the start of the experiment. The breast cancer cells seem to migrate as a result of increasing EGF concentration.

To study the effect of EGFR overexpression on cell migration, ZR11 (EGFR-transfected) and ZR9b11 (mock-transfected) breast tumor cells were incubated with various EGF concentrations dissolved in 100 μl agarose in the presence of 10% serum. After 48 h the number of migrated cells was determined (Fig. 2A). ZR9b11 cells showed only a slight increase in cell migration (up to 1.9-fold), whereas ZR11 cells showed an increase of approximately 20-fold. Optimal migration was observed by incubation with 5 ng EGF/100 μl. Al-

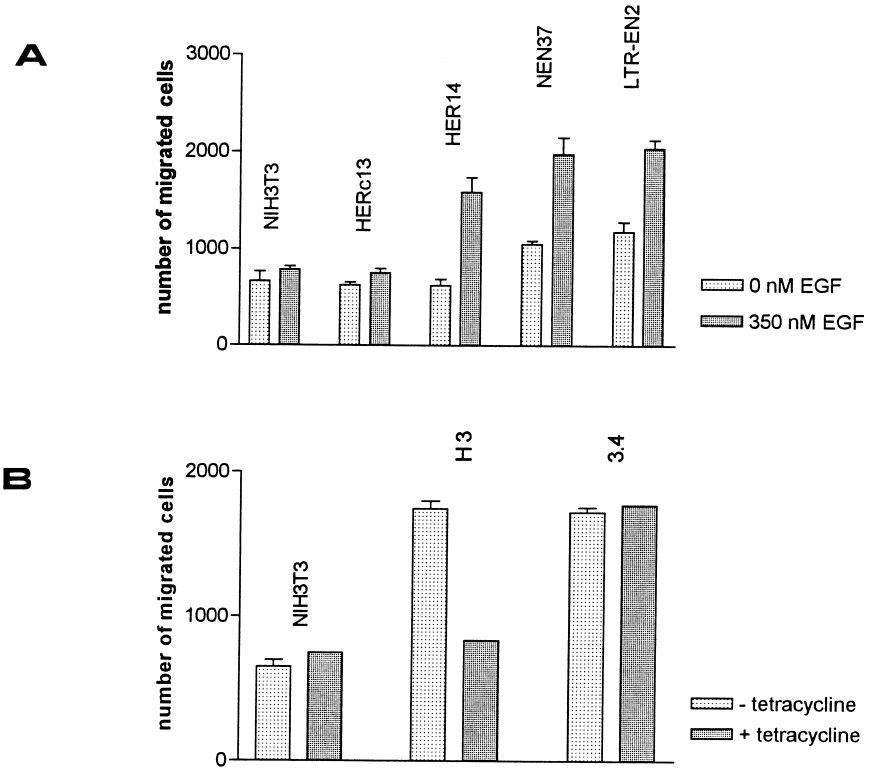

Fig. 3. Cell migration of EGFR and c-erbB2 overexpressing NIH3T3 fibroblasts. A: Cells (as indicated) were incubated with 0 or 5 ng EGF/100 μl agarose. After 48 h the number of migrated cells was counted in 10 random fields under a phase-contrast microscope (100× magnification). Representative experiment out of three, performed in triplicate (mean ± S.D). B: NIH3T3, H3 and 3.4 cells were incubated with tetracycline (96 h, 1 μg/ml, H3 cells show no c-erbB2 expression) or not (H3 cells overexpress c-erbB2). After 48 h the number of migrated cells was counted in 10 random fields under a phase-contrast microscope (100× magnification). Representative experiment out of two, performed in triplicate (mean ± S.D.).

most no difference in cell migration was seen between unstimulated ZR9b11 and ZR11 cells, indicating that EGFR overexpression alone is not sufficient to induce cell migration.

To confirm that this cell migration was indeed the effect of EGF stimulation of the EGFR, we preincubated ZR11 cells for 2 h with two different EGFR antibodies (Ab-1, Calbiochem, Cambridge, MA and Ab-2, Oncogene, Manhasset, NY, recognizing different epitopes in the external domain, and interfering with ligand binding). Preincubation with these anti-EGFR antibodies decreased the number of migrated ZR11 cells (Fig. 2B), indicating that activation of EGFR by binding of its ligand is necessary for its effect on migration of human breast cancer cells.

Activation of the EGFR leads to phosphorylation of its C-terminus. Several SH2 domain (Src homology)-containing proteins have been found to bind to the phosphotyrosine residues of the receptor. These proteins activate different pathways that result in cell proliferation, differentiation, motility, and adhesion [24]. In an attempt to unravel the pathway that is involved in migration of the human breast cancer cells, we preincubated ZR11 cells with a series of inhibitors. We used inhibitors of receptor tyrosine kinases, c-Src tyrosine kinases, and tyrosine phosphatases. Unfortunately, incubation with high concentrations of these inhibitors resulted in cell death. In contrast, using inhibitors for MAPKK (PD 98059, EI-360, Biomol, Plymouth, PA) and PI3K (LY 294002, ST-420, Biomol or wortmannin, ST-415, Biomol), no cell death was detected. Preincubation of ZR11 cells with the MAPKK inhibitor (10 μM, 2 h) resulted in a decrease in cell migration (2-fold, Fig. 2B). No influence on ZR9B11 migration was found (not shown). Incubation with this inhibitor did indeed result in hypophosphorylation (and inactivation) of MAPK, as was confirmed by MAPK shift assays (data not shown). These results indicate that MAPK activation might have a positive effect on cell migration induced by EGFR activation.

When ZR11 cells were preincubated with the PI3K inhibitors (LY 10 μM or wortmannin 100 μM, 2 h) EGF-induced cell migration was stimulated approximately 2-fold (Fig. 2B). Again, no influence on migration of ZR9b11 cells was found. Therefore, in contrast to MAPK, PI3K appears to have an inhibiting effect on migration of ZR11 cells in this system. Furthermore, in the absence of EGF the inhibitors had no effect on cell migration.

To investigate whether the effect of EGFR overexpression was cell type-specific, we also used NIH3T3 fibroblasts, HERc13 and HER14 (both transfected with wild type EGFR [20], Table 1) in the migration assay. In these assays we used non-ECM-coated filters in the Transwell system, since the migration of fibroblasts was negligible using coated filters. Fig. 3A shows that the cell migration of fibroblasts overexpressing EGFR is also increased upon EGF incubation. Migration of HER14 cells, with the highest amount of EGFR, was increased approximately 2.6-fold by EGF addition, whereas migration of HERc13 cells showed hardly any increase.

Overexpression of c-erbB2 is, like EGFR, often found in human breast cancer. Since the ligand of c-erbB2 has not been identified so far, we used chimerae of EGFR/c-erbB2 that can

Table 1
EGFR and c-erbB2 expressing cell lines

Cell line	Expression of	Receptors/cell	Reference
ZR9b11	EGFR	20 000	[20]
ZR11	EGFR	1 200 000	[20]
NIH3T3	EGFR	3 000	ATCC
HERc13	EGFR	65 000	[21]
HER14	EGFR	250 000	[21]
NEN37	EGFR/c-erbB2	400 000	[22]
LTR-EN2	EGFR/c-erbB2	1 400 000	[22]
H3	c-erbB2	~500 000	[23]
3.4	c-erbB2	~2 000 000	[23]

be stimulated with EGF (NEN37 and LTR-EN2 [21], Table 1) in our migration assay. Fig. 3A shows that even without EGF incubation these cells migrate better than control NIH3T3 cells. Upon EGF incubation, migration of NEN37 cells was increased 1.9-fold and migration of LTR-EN2 cells 1.7-fold.

To study whether c-erbB2 overexpression alone was sufficient for induction of cell migration we used H3 cells (NIH3T3 cells expressing c-erbB2 under a tetracycline-responsive promoter [23], Table 1). By addition of tetracycline the expression of c-erbB2 disappears. This was confirmed by incubation of H3 cells with tetracycline (1 μg/ml) for different time periods, immunoprecipitating c-erbB2 from cell lysates, and Western blotting experiments using anti-c-erbB2 antibodies. After 48 h of tetracycline incubation no c-erbB2 protein could be detected ([23], and own observation). Additionally, we used 3.4 cells that constitutively express c-erbB2 ([23], Table 1). Our results show that overexpression of c-erbB2 alone does indeed increase cell migration of NIH3T3 cells (Fig. 3B). Without tetracycline incubation H3 cells express c-erbB2 and show as much cell migration as the 3.4 cells. After incubation with tetracycline (48 h preincubation and during the assay) H3 cells do not express c-erbB2 any more and, as a consequence, cell migration is comparable with NIH3T3 cells. Addition of tetracycline to NIH3T3 cells or 3.4 cells did not affect cell migration (Fig. 3B).

In conclusion, our results show that overexpression of EGFR and c-erbB2 induces cell migration in our in vitro model of cell migration. The effect of EGFR is only found upon activation of the receptor by EGF, while overexpression of c-erbB2 alone appears to be sufficient. Furthermore, in the human breast cancer cells both MAPK and PI3K might be involved in cell migration induced by the EGFR.

4. Discussion

In breast cancer, as in most other solid tumors, the metastatic phase of the disease rather than the primary lesion is the cause of death of the majority of patients [24]. In this report we showed that overexpression of EGFR and c-erbB2, both prognostic factors, can induce cell migration, an important step in the metastatic cascade. It has been reported that in vitro invasiveness correlates with the in vivo metastatic potential of tumor cells [25].

We found that when EGF was used as a chemoattractant, the cell migration of EGFR and EGFR/c-erbB2 chimera expressing cells was increased. Increased expression of EGFR in metastases of human breast cancer, as compared to cells of the primary tumor, suggests a contribution of EGFR to breast cancer metastasis [26]. To test a causative link between expression of EGFR and the process of metastasis Lichtner et al. [27] introduced and expressed full length cDNA for the human EGFR in low metastatic MTC rat mammary adenocarcinoma cells. These cells respond to EGF by enhanced matrix adhesion and increased lung colonizing potential.

In our assays breast cancer cells migrate much better than NIH3T3 fibroblasts after addition of EGF, suggesting fundamental differences in the intracellular events that follow the binding of EGF to its receptor on epithelial cells and fibroblasts. Also, the magnitude of the effect may be directly related to the amount of EGFR expression.

We showed that both the MAPK and PI3K pathways are involved in EGF-induced cell migration in the breast cancer cells. It has been described that upon PDGF activation MAPK influences the cell motility by phosphorylating and thereby enhancing myosin light chain kinase activity. This leads to phosphorylation of myosin light chains and polymerization of actin cables, resulting in enhanced cell migration [28]. Our results suggest that in human breast cancer cells activation of MAPK by EGFR may lead to increased cell migration via similar pathways. MAPK is often implicated in the Ras pathway leading to cell proliferation and differentiation. However, mitogenic and motogenic signals may be on a common pathway to MAPK activation and then diverge based an the ability of MAPK to translocate to distinct intracellular compartments [29]. Indeed, MAPK has been shown to translocate to the nucleus [29] as well as to the cytoskeleton [30].

We found that PI3K has a negative effect on EGF-induced migration of human breast cancer cells. It has been described that activation of PI3K increases cell matrix adhesion. Upon stimulation of the PDGF receptor, PI3K transmits signals that activate integrin adhesiveness [31]. The p85 subunit of PI3K has been found to associate, in a integrin-dependent manner, with tyrosine phosphorylated FAK [32]. In the Transwell system, increased cell matrix adhesion may very well lead to decreased cell migration, since we used ECM-coated filters.

Our data indicate that overexpression of c-erbB2 is sufficient to induce cell migration. From the literature it is known that overexpression of c-erbB2 in NIH3T3 cells mediates transformation [33]. A critical level of c-erbB2 overexpression seems to be necessary to achieve transformation [34]. This phenomenon can be explained by a model in which there is an equilibrium between monomeric and oligomerized forms of c-erbB2. As the quantity of c-erbB2 protein increases by overexpression, the equilibrium is shifted to the oligomeric state,

resulting in activation of the tyrosine kinase and inappropriate cellular signaling [35].

The process of cell migration requires the coordinated activation of both growth factor and adhesion receptor signaling. It has been described that signals downstream of EGFR and c-erbB2 can modulate integrin-mediated processes in both an inhibitory and a stimulatory manner [8]. Since the observed patterns of tyrosine phosphorylated proteins in NIH3T3 cells transformed by EGFR or c-erbB2 are qualitatively and quantitatively different [36], further studies will be required to unravel the pathways that lead from either EGFR or c-erbB2 overexpression to metastatic spread of tumor cells.

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