Published in final edited form as:

Cancer Lett. 2012 December 30; 326(2): 183–190. doi:10.1016/j.canlet.2012.08.013.

An in vivo model of epithelial to mesenchymal transition reveals a mitogenic switch

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Abstract

The epithelial to mesenchymal transition (EMT) is a process by which differentiated epithelial cells transition to a mesenchymal phenotype. EMT enables the escape of epithelial cells from the rigid structural constraints of the tissue architecture to a phenotype more amenable to cell migration and, therefore, invasion and metastasis. We characterized an in vivo model of EMT and discovered that marked changes in mitogenic signaling occurred during this process. DNA microarray analysis revealed that the expression of a number of genes varied significantly between post-EMT and pre-EMT breast cancer cells. Post-EMT cancer cells upregulated mRNA encoding c-Met and the PDGF and LPA receptors, and acquired increased responsiveness to HGF, PDGF, and LPA. This rendered the post-EMT cells responsive to the growth inhibitory effects of HGF, PDGF, and LPA receptor inhibitors/antagonists. Furthermore, post-EMT cells exhibited decreased basal Raf and Erk phosphorylation, and in comparison to pre-EMT cells, their proliferation was poorly inhibited by a MEK inhibitor. These studies suggest that therapies need to be designed to target both pre-EMT and post-EMT cancer cells and that signaling changes in post-EMT cells may allow them to take advantage of paracrine signaling from the stroma in vivo.

Keywords

PDGF; HGF; EMT; c-Met; Her2/neu

1. Introduction

EMT is a pivotal switch in breast cancer progression. During this transition, breast cancer cells transform from an epithelial to a more migratory, mesenchymal-like phenotype, which is associated with increased motility and invasiveness. Ultimately, these cells metastasize [1,2].

Injection of a MMTV-Her2/neu breast cancer cell line into syngeneic mice results in tumors that undergo EMT in vivo [3]. We employed a similar approach to generate pre- and post-EMT MMTV Her2/neu breast cancer cell lines to examine differences in gene expression in

these cells. Here we show that in vivo EMT of MMTV-Her2/neu breast cancer cells is associated with marked changes in receptor tyrosine kinase expression and alterations in signaling through downstream mitogenic cascades. In addition to acquiring responsiveness to PDGF, the post-EMT cells also acquired enhanced responsiveness to hepatocyte growth factor (HGF) and lysophosphatidic acid (LPA), and exhibited constitutive tyrosine phosphorylation of the receptor tyrosine kinase Axl and the transcription factor STAT3. The post-EMT cells were less sensitive than the pre-EMT cells to MEK inhibitor U0126, but more sensitive to the growth inhibitory effects of PDGF, HGF, and LPA receptor inhibitors/antagonists.

Human breast cancer cell lines showed analogous changes in mitogenic protein expression correlating with their EMT status. Inducing a mesenchymal appearance in a normal epithelial cell line, MCF10A, by growth in medium supplemented with 10% fetal bovine serum rather than with the traditional supplements of 5% horse serum, EGF, hydrocortisone, insulin, and cholera toxin [4] caused changes in the expression of EMT markers and mitogenic signaling proteins including PDGFR β and Axl. Likewise, treatment of MCF10A cells with TGF- β , which induces a mesenchymal appearance along with an increase in invasiveness dependent on an upregulation of EGFR [5], also caused changes in the expression of EMT markers and mitogenic signaling proteins including PDGFR β and Axl.

2. Materials and methods

2.1. In vivo EMT model

The previously described neuT cancer cell line [6,7] was injected (10^6 cells) into the inguinal (#4) mammary fat pads of wild type FVB mice. Tumors were allowed to grow to between 1 and 1.5 cm in diameter to permit the tumors to undergo EMT and the tumors were harvested and clonal cancer cell lines were isolated as described previously [6,7].

2.2. Cell culture

Human cancer cell lines were purchased from ATCC (Manassas, VA). Unless otherwise indicated, all cell lines were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum. Stable knockdown cell lines were generated by co-transfecting shRNA constructs (Thermo Scientific, Waltham, MA) along with viral packaging plasmids PMD2G and PsPax2 obtained from Addgene (Cambridge, MA) into the 293T cell line using Lipofectamine Reagent (Invitrogen, Grand Island, NY). Medium from the transfected 293T cell line was then used to infect the target cell line, which was subsequently selected using 10 μ g/mL Puromycin. The MMTV-D1K2-T2 cell line was described previously [7].

2.3. Microarray analysis

Total RNA was isolated from neuT luminal and neuT_{EMT,CL2} cells using Trizol Reagent (Invitrogen), according to the manufacturer's instructions. Three replicate samples of RNA from each cell line were isolated and analyzed. Microarray analysis was performed using the Affymetrix microarray platform at the Interdisciplinary Center for Biotechnology Research (ICBR) Microarray Core, University of Florida. Total RNA concentration was determined with a NanoDrop Spectrophotometer (Nano-Drop Technologies, Inc., Wilmington, DE), and sample quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). All microarray sample preparation reactions used the GeneChip[®] Whole Transcript (WT) Sense Target Labeling reagents (Affymetrix, Inc., Santa Clara, CA), and reactions were performed following the manufacturer's protocols. The arrays were washed and stained with the reagents supplied in the GeneChip[®] Hybridization Wash and

Stain kit (Affymetrix, Inc.) on an Affymetrix Fluidics Station 450, and scanned with a GeneChip® 7G Scanner (Affymetrix, Inc.).

2.4. Immunoblot analysis

Cell lysates were prepared as described previously [6,7] and were analyzed by immunoblot with the following antibodies: [Actin (sc-1616-R), Erk1/2 (sc-93), Gas6 (sc-1936), IRS-1 (sc-559), N-Cadherin (sc-7939), PDGFR β (sc-432), SPARC (sc-25574), Stat3 (sc-7179); Santa Cruz, Santa Cruz, CA], [Akt (#9272), Ax1 (#4977), c-Met (#3127), p-Akt S473 (#9271S), p-Akt T308 (#9275S), p-Ax1 (#5724P), p-Erk T202/Y204 (#9101S), p-Met Y1234/12345 (#3126), p-PDGFR β Y751 (#4549), p-Stat3 Y705 (#9131S), Zeb1 (#3396S); Cell Signaling, Danvers, MA], [Her2 (MS-730-P0), Vimentin (MS-129-P0); Neomarkers, Fremont, CA], [E-Cadherin (610181); BD Transduction, San Jose, CA], [LPA1 (10005280); Cayman Chemical Company, Ann Arbor, Michigan], [Occludin (711500); Invitrogen Carlsbad, CA].

2.5. Proliferation assays

Proliferation was measured using tritiated thymidine incorporation assays as described [6,7]. When assessing proliferation induced by HGF, LPA, or PDGF, these factors were applied to the cells diluted in 1x Insulin, Transferrin, and Selenium (ITS) (Roche, Branchburg, NJ). Results are presented as the average $\pm$ standard deviation of triplicate determinations.

2.6. Invasion assays

Cells were plated in the inserts of 6-well BD BioCoat Matrigel Invasion Chamber plates (#354480 BD, San Jose, CA) at 125,000 cells per chamber in 0.2% FBS-DMEM. Serum (10% FBS) was added to the lower chambers to act as a chemoattractant. Cells were incubated at 37 °C for 48 h. Following incubation, non-invaded cells were removed from the inserts with cotton swabs. The remaining invaded cells were fixed with ice-cold methanol for 10 min, and stained with 0.5% crystal violet/25% methanol for 10 min. Invaded cells were counted in three or more randomly chosen fields. Results are presented as the average $\pm$ SD of triplicate determinations.

2.7. MCF10A EMT models

Unless otherwise stated, MCF10A cells were maintained in a 50/50 mixture of Dulbecco's Modified Eagle's Medium and Ham's F12 medium supplemented with 5% horse serum, 20 ng/mL EGF, 100 ng/mL cholera toxin, 10 μ g/mL insulin, and 500 ng/mL hydrocortisone (Sigma, St. Louis, MO). For induction of EMT, cells were grown in 50/50 DMEM/F12 + 10% fetal bovine serum or in the presence of 2.5 ng/mL TGF- β (Millipore, Billerica, MA) for a minimum of 4 weeks prior to photographing and processing.

3. Results

3.1. In vivo EMT as a function of tumor volume

We previously described a cell line derived from a transgenic mouse MMTV-Her2/neu tumor, hereafter referred to as neuT [6,7]. NeuT cells are capable of forming tumors with sharp boundaries defined by a capsule and exhibit high levels of E-Cadherin that form functional adherens junctions. In subsequent studies it was observed that when the tumors reach approximately 1 cm in diameter the morphology of the cancer cells changes from a cuboidal shape with little cytoplasm to one in which the cells were elongated and have abundant cytoplasm. Coincident with this change in morphology, the tumors lost their defined border and incorporated inclusions of adipocytes and muscle as they invaded into surrounding tissues (Fig. 1A). These alterations were consistent with EMT. Cell lines were

isolated from large tumors that had undergone this transition and were subjected to microarray analysis to compare their expression profiles to that of the parental neuT cell line (Table 1 and Supplementary Fig. S1). The observed patterns in mRNA expression were consistent with EMT, and expression of several of the corresponding proteins changed in a manner in agreement with the microarray analysis (Fig. 1B and Supplementary Fig. S2). In contrast to the neuT cells, the clonal post-EMT cell lines expressed the mesenchymal markers N-Cadherin, Zeb1, and SPARC [8–11], and exhibited decreased expression of the epithelial markers E-Cadherin and Occludin. PCR analysis showed that the post-EMT cells harbored the rat Her2/neu transgene, confirming that they were derived from the original neuT cell line (Supplementary Fig. S3).

The cells grown in culture maintained a morphology that was consistent with that observed in tumors in vivo. In contrast to the pre-EMT cells, the post-EMT cells did not readily form colonies in culture (Fig. 1C) and the post-EMT cells exhibited an enhanced ability to invade through Matrigel-impregnated membranes (Fig. 1D). The invasive properties of the post-EMT cells were apparent when grown as tumors in vivo by the lack of a capsule and defined border, and extensive invasion through the adjacent mammary fat pad (Fig. 1E). This occurred at a small tumor size (6 mm) at which tumors derived from the pre-EMT neuT cells have not yet undergone EMT and acquired this ability.

3.2. Altered expression of mitogen receptors and mitogen responsiveness occurs during EMT

An interesting finding from the microarray analysis was that several of the genes whose expression was altered during EMT in vivo are those encoding receptor tyrosine kinases (RTKs) and other signaling molecules (Table 1). We pursued this observation further because such changes will likely have important effects on tumor responsiveness to therapeutic agents that target these signaling intermediates. Immunoblot analyses indicated that Her2 and IRS-1 expression were decreased following EMT, and EMT was associated with increased expression of the RTKs Axl, PDGFR, and c-Met (Fig. 2A). This is consistent with previous observations associating these receptors with EMT [12–15]. We also observed an increased level of c-Met phosphorylation on Tyr^{1234/1235} and constitutive activating phosphorylation of the transcription factor STAT3 on Tyr⁷⁰⁵, while PDGFR phosphorylation could not be detected (data not shown).

Experiments were performed to determine the functional consequences of these EMT-associated changes in expression. The microarray data also revealed increased mRNA levels of the lysophosphatidic acid receptor LPAR1 in the post-EMT cells. Increasing concentrations of the mitogen LPA dose-dependently increased the proliferation of the post-EMT cells, but had a limited effect on the pre-EMT cells (Fig. 2B). The mitogenic effects of LPA, and to a lesser extent serum, were inhibited by the LPAR1 and 3 antagonist Ki-16425 (Fig. 2C). Similarly, HGF and PDGF stimulated the proliferation of the post-EMT cells to a significantly greater extent than that of the pre-EMT cells (Fig. 3A and C). These effects were blocked by SU11274 and Gleevec, inhibitors of the tyrosine kinase activity of c-Met and PDGFR, respectively (Fig. 3B and D).

To examine the role of basal signaling in the absence of exogenous ligand, thymidine incorporation experiments were carried out in which pre- and post-EMT neuT cells grown in $1 \times \text{ITS}$ were treated with Ki-16425, SU11274, or Gleevec (Supplementary Fig. S4). Inhibition of LPAR or c-Met resulted in an appreciable decrease, 60–80%, in proliferation in both cell lines, indicating that a basal level of activity may exist in these pathways. Inhibition of PDGFR caused a more modest, 30% decrease in proliferation. This is in agreement with the lack of PDGFR phosphorylation observed in the absence of exogenous

PDGF. The observed cellular inhibition could be due to off-target effects of the tyrosine kinase inhibitor.

3.3. EMT is associated with changes in cellular signaling cascades and sensitivity to targeted therapeutic agents

The marked changes in growth factor receptor expression during EMT suggests that EMT is also associated with alterations in downstream signaling cascades and cancer cell responsiveness to therapeutic agents that target these pathways. The post-EMT cell lines exhibited diminished levels of steady-state Akt and Erk phosphorylation on activating sites (Fig. 4A). The decreased Erk and Raf phosphorylation suggested that pre- and post-EMT cells might have differential sensitivity to a MEK inhibitor. Fig. 4B shows that the pre-EMT cells are significantly more sensitive to MEK inhibitor U0126 than the post-EMT cells, suggesting that the pre-EMT cells are more dependent on signaling through the Ras/Erk pathway for their proliferation.

Further, immunoblot analysis was carried out on pre- and post- EMT cells treated with the PI3K inhibitor, LY294002, or U0126 in the absence of exogenous ligand or with PDGF, HGF, or LPA in order to examine the effects of these treatments on downstream signaling events. U0126 treatment almost completely abrogated basal and growth factor-induced Erk phosphorylation in both the neuT and neuT_{EMT, CL2} cell lines (Fig. 4C). These data are in agreement with Fig. 4A and 4B and suggest that the increased sensitivity of the pre-EMT cell line to U0126 may be due to its increased basal activation of the Erk pathway. LY294002 blocked AKT phosphorylation caused by PDGF or HGF, demonstrating the role of the PI3K signaling cascade. Fig. 4C also illustrates that while there is a lack of basal PDGFR phosphorylation in both pre- and post-EMT cell lines, only the cells that have undergone EMT and display increased PDGFR expression are able to activate PDGFR signaling, marked by receptor phosphorylation, in the presence of exogenous PDGF.

3.4. Basal-like human breast cancer cell lines upregulate c-Met, PDGFR, and Axl

EMT is related to the basal-like subtype in human breast cancers [11]. The basal-like subtype in turn overlaps with the triple-negative category of breast tumors that lack Estrogen Receptors, Progesterone Receptors, and Her2 [16–18]. Therefore we examined whether any of the molecular alterations associated with EMT in the neuT mouse model were also present across a panel of human breast cancer cell lines of known subtype based on microarray analysis [19]. An inverse correlation was observed between E-Cadherin levels and the expression of Axl, the Axl ligand GAS-6, and c-Met (Fig. 5A). However expression of Axl, GAS-6, and c-Met partially overlapped with that of the mesenchymal markers Vimentin, Zeb1, and SPARC. In contrast to the neuT EMT model, there was a reciprocal expression of c-Met and PDGFR in the MDA-MB 157 post-EMT cell line (Fig. 5A, right half of breast panel). MDA-MB-231, a post-EMT cell line, expressed high levels of GAS-6 and exhibited strong Axl phosphorylation.

We previously showed that E-Cadherin function is dysregulated in MDA-MB-231 cells [20] and hypothesized that blocking Axl signaling might restore E-Cadherin function, given the role of Axl in inducing EMT and increasing invasion [12]. Stable Axl knockdown did not alter E-Cadherin localization or function (data not shown). Interestingly, GAS-6 knockdown blocked Axl phosphorylation, demonstrating that MDA-MB-231 cells have a GAS-6/Axl autocrine loop that may contribute to the aggressive behavior of these cells. Together, these observations in human breast cancer cell lines indicate that upregulation of Axl in the mouse breast cancer cells undergoing EMT is relevant to processes that occur during the progression of human breast cancer.

3.5. In vitro models of EMT show analogous mitogenic changes

Similar changes in mitogenic signaling were observed in two models of EMT in MCF10A cells. MCF10A, nontransformed human mammary epithelial cells, have a distinctly cuboidal, epithelial morphology when grown in their recommended medium: 50/50 DMEM/F12 supplemented with 5% horse serum, 20 ng/mL EGF, 100 ng/mL cholera toxin, 10 µg/mL insulin, and 500 ng/mL hydrocortisone (Fig. 6A left panel). When MCF10A cells were grown in 50/50 DMEM/F12 + 10% fetal bovine serum (FBS), the cells assumed a mesenchymal morphology characterized by flattened cells and a lower tendency to form cell-cell contacts (Fig. 6A right panel). Immunoblot analyses of cells maintained in both growth media (Fig. 6B) showed a clear change in the expression of EMT markers, including a switch from E-Cadherin to N-Cadherin and increased expression of Vimentin and Zeb1. Consistent with the models discussed previously, the cells also displayed increased expression of PDGFR β and Axl and showed a higher level of Axl phosphorylation. The observed decrease in c-Met expression is likely due to the absence of added EGF in the 10% FBS medium, which has been shown to upregulate c-Met expression [21].

Similarly, addition of 2.5 ng/mL TGF- β to complete MCF10A medium induced the cells to undergo EMT, as evidenced by a morphological change (Fig. 6C), increased Vimentin and Zeb1 expression, and a partial cadherin switch (Fig. 6D). These cells also showed higher levels of PDGFR β and Axl. The minor increase in c-Met expression mirrors the neuT tumor model.

4. Discussion

The investigation of EMT in cancer has primarily focused on this process as a means by which cancer cells acquire the ability to separate from the parent tumor mass, invade locally, and metastasize to distant sites. EMT has been extensively studied in cell culture systems and is induced by diverse growth factors and cytokines including HGF, TGF- β , TNF α , IL6, and PDGF and combinations thereof [22–28]. Each of these factors stimulate different subsets of signaling pathways, making it difficult to dissociate the effects of these factors themselves from changes associated with EMT. Previous work has shown that injection of MMTV-Her2/neu cancer cells into mice that are syngeneic, with the exception of the neu expression in the cancer cells, results in tumors that undergo EMT in vivo [3]. The present results obtained using an in vivo system may be more physiologically relevant than those obtained using in vitro systems because in this model EMT occurs in vivo in an orthotopic setting and in an immune-competent background.

We employed a similar approach to generate pre- and post-EMT MMTV-Her2/neu breast cancer cell lines to study changes in cell signaling that occur during EMT. Microarray analysis indicated that in addition to alterations in the expression of EMT-associated proteins, numerous changes in the expression of elements of mitogenic signaling cascades were also observed (Table 1, and Supplementary Figs. S1 and S2). Interestingly, several of the EMT-associated changes in the levels and phosphorylation state of signaling molecules have been implicated either as markers for EMT or as inducers of EMT, including Axl, PDGFR, c-Met, and STAT3 phosphorylation [12–30]. Importantly, many of these changes also correlate with the expression of EMT markers in human breast cancer cell lines (Fig. 5).

PDGF and PDGFR are upregulated in several models of EMT. For instance, TGF- β and hyperactive Ras synergize to induce EMT in hepatocytes [28]. In this model of hepatocellular EMT, the expression of PDGF-A ligand and both the α and β PDGF receptor subunits are highly elevated upon EMT, thus producing a PDGF autocrine loop. Similarly, TGF- β induces a PDGF autocrine loop in MMTV-Neu x MMTV-TGF- β double transgenic mice, and the Rastransformed murine mammary epithelial cell line Eph4 (EpRas)

undergoes TGF- β induced EMT with a concomitant establishment of a PDGF autocrine loop and hyperactivation of PI3K [15]. Our model differs in that the MMTV-Her2/neu cancer cells used for injection into mice were not engineered to overexpress TGF- β although it is possible that these cells [7], or the tumor microenvironment provided sufficient amounts of TGF- β to induce EMT.

Alterations in baseline signaling through mitogenic pathways that occur as a consequence of EMT likely influence how tumor cells respond to targeted anticancer therapeutic agents. Post-EMT neuT cells, for example, exhibited decreased basal Akt, Raf, and Erk phosphorylation, and in comparison to the pre-EMT cells, their proliferation was poorly inhibited by the MEK inhibitor U0126 (Fig. 4A and B), which was able to eliminate the high basal level of pre-EMT Erk phosphorylation (Fig. 4C). Our model further differed from those cited above since we did not observe hyperactivation of PI3K. It may therefore be important to analyze the PI3K and Erk status of a post-EMT cancer before utilizing treatments that target these signaling pathways.

Increased responsiveness to HGF and LPA was observed in the post-EMT neuT cell lines. It is interesting that both of these factors increase cell motility and invasiveness [31,32], which are traits associated with EMT. This is the first report, to our knowledge, that post-EMT cells become more responsive to LPA. This is an important observation because increased LPAR1 mRNA expression in primary tumors of breast cancer patients correlates significantly with their positive lymph node status [33]. Furthermore, an LPAR antagonist reduces breast cancer cell migration and invasion in vivo [34]. Additional studies are required to determine to what extent the HGF- and LPA-dependent signaling pathways contribute to EMT and its maintenance, and the motile, invasive properties associated with cells that have undergone EMT.

Tumor cells in vivo may utilize changes in mitogenic signaling in an autocrine (basal) or paracrine manner. Our data suggest that both pre- and post-EMT cells have a basal level of LPAR and c-Met activation, but that the post-EMT cells have a much more dramatic increase in proliferation in response to exogenous LPA or HGF. There was little basal PDGFR activation in either cell line, however, the post-EMT cell lines showed a robust response to the addition of PDGF. These cells increased proliferation and exhibited PDGFR phosphorylation, whereas the pre-EMT cells showed little or no response to PDGF. Therefore, the mitogenic changes associated with EMT may allow post-EMT tumor cells to better exploit growth factors released by the stroma.

It is interesting to note that STAT3 activation increases in the post-EMT MMTV-Her2/neu breast cancer cell lines and in the cells that have undergone EMT in the breast panel. This is not unexpected since STAT3 is activated by an array of cytokines, growth factors (including EGF and PDGF), and some G-protein coupled receptor agonists [35–37]. PDGF or other growth factors or cytokines might activate STAT3 in the post-EMT cells, and this is a subject for future study.

In summary, we characterized a reproducible in vivo model of EMT and find that striking changes in mitogenic signaling take place during this transition. Similar alterations are associated with EMT in a panel of human breast cancer cell lines as well as in normal human mammary epithelial cells grown in media that induce a mesenchymal phenotype. These changes alter the responsiveness of the cancer cells to both growth factors and to compounds that selectively inhibit their respective downstream signaling pathways. Therapeutic agents targeting c-Met, Axl, and STAT3, are currently under development [38–42], and have the potential to attack the most aggressive breast tumors. Additional studies are required to fully determine the consequences of these observations with respect to the

treatment of breast cancer patients with drugs targeting Her2, PDGFR, LPAR, c-Met, and STAT3, and the use of these agents in combination therapies that simultaneously target pre- and post-EMT cancer cells within the same tumor. Further, the observation that tumorigenicity can be maintained in cancer cells initiated by Her2 expression despite loss of detectable Her2/neu expression, presumably through “mitogenic switching,” may have important implications for the acquisition of tumor resistance to Her2-targeted therapeutic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by National Institutes of Health Grant R01-CA93651, Bankhead-Coley Grant 09BB-10, and Susan G. Komen Grant KG080510 (to B.L.).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.canlet.2012.08.013>.

Fig. 1.

Characterization of an in vivo model of EMT. (A) Micrographs of hematoxylin and eosin (H&E) stained tumors of the indicated sizes formed from neuT cells. Red arrows point out cancer tissue and cell morphology. Yellow arrows denote tumor boundaries. Black arrows indicate fibroblast capsules. Green arrows show inclusions of adipocytes or muscle tissue in the tumors. (B) The original neuT cell line and clonal tumor cell lines derived from neuT cells after having undergone EMT in vivo (neuT_{EMT,CL2} and neuT_{EMT,CL5}) cultured in DMEM with 10% FBS and analyzed by immunoblot with the indicated antibodies. Actin served as a loading control. (C) Phase contrast micrographs showing cell morphology of neuT and neuT_{EMT,CL2} cells growing in culture. Insets show cells at higher magnification. (D) Invasion assays quantifying the number of cells per field permeating a Matrigel impregnated filter. (E) Micrograph of an H&E stained 6 mm tumor formed from neuT_{EMT,CL2} cells. Lower panels are higher magnification images showing cancer cell infiltration around mammary adipocytes and ducts.

Fig. 2.

EMT is associated with altered growth factor receptor expression and responsiveness to lysophosphatidic acid. (A) Immunoblots of extracts prepared as described in Fig. 1B were analyzed with the indicated antibodies. (B) The neuT and neuT_{EMT,CL2} cells were stimulated with the indicated concentrations of lysophosphatidic acid (LPA) for 24 h and ³H-thymidine incorporation was measured. (C) The neuT and neuT_{EMT,CL2} cells were stimulated with 10 μM LPA or 10% FBS in the presence of the indicated concentrations of Ki-16425 for 24 h and ³H-thymidine incorporation was quantitated.

Fig. 3.

Post-EMT cells exhibit enhanced responsiveness to the mitogenic actions of HGF and PDGF. (A) The neuT, neuT_{EMT,CL2}, and neuT_{EMT,CL5} cell lines were stimulated with the indicated concentrations of HGF for 24 h and cell proliferation was measured by ^3H -thymidine incorporation. (B) The neuT, neuT_{EMT,CL2}, and neuT_{EMT,CL5} cell lines were stimulated with 10 ng/ml HGF in the presence of the indicated concentrations of SU11274 for 24 h and ^3H -thymidine incorporation was quantitated. (C) The neuT, neuT_{EMT,CL2}, and neuT_{EMT,CL5} cell lines were stimulated with the indicated concentrations of PDGF for 24 h and ^3H -thymidine incorporation was quantitated. (D) The neuT, neuT_{EMT,CL2}, and neuT_{EMT,CL5} cell lines were stimulated for 24 h with 10 ng/ml PDGF in the presence of the indicated concentrations of Gleevec for 24 h and ^3H -thymidine incorporation was quantitated.

Fig. 4.

EMT is associated with changes in intracellular signal transduction cascades. (A) Immunoblot analysis of extracts prepared as in Fig. 1B were analyzed with the indicated antibodies. (B) The neuT, neuT_{EMT,CL2}, and neuT_{EMT,CL5} cell lines were treated with the indicated concentrations of U0126 in 10% FBS-DMEM growth medium for 24 h and ³H-thymidine incorporation was quantitated. (C) Immunoblot analysis of cell lysates from neuT and neuT_{EMT,CL2} cells pre-incubated in serum free medium. Cells were pre-treated with inhibitors for 1.5 h and then co-treated with the inhibitors and growth factors for 30 min prior to analysis.

Fig. 5.

Expression of EMT markers in human breast cancer cell lines. (A) Immunoblot analysis of human breast cancer cell lines with the indicated antibodies. (B) Immunoblot analysis of a MDA-MB-231 cell line stably expressing an E-Cadherin-green fluorescent protein fusion protein (231-E-Cad-GFP) transduced with lentiviral vectors encoding short hairpin RNAs targeting Axl, GAS6, or a scrambled control shRNA.

Fig. 6. EMT of MCF10A cells is associated with altered expression of growth factor receptors. (A) Micrographs of MCF10A cells grown in MCF10A complete medium or DMEM/F12 + 10% FBS. (B) Immunoblot analysis of MCF10A cells grown in MCF10A complete medium or DMEM/F12 + 10% FBS. (C) Micrographs of MCF10A cells grown in MCF10A complete medium with or without 2.5 ng/mL TGF- β . (D) Immunoblot analysis of MCF10A cells grown in MCF10A complete medium with or without 2.5 ng/mL TGF- β . The arrow denotes the p-Axl band, while the “*” marks a non-specific band.

Table 1

Changes in gene expression in pre- versus post-EMT cells.

<i>Upregulated during EMT in vivo (*upregulation verified by immunoblot)</i>			
Gene symbol	GeneID	Fold-upregulated	Localization/function
Axl*	26362	91	Receptor tyrosine kinase
Cadherin 2 (N-Cadherin)*	12558	7.8	Cell-cell adhesion
LPAR1	14745	7.4	Lysophosphatidic acid receptor
Met*	17295	2.5	Receptor tyrosine kinase
PDGFRa	18595	8.7	Receptor tyrosine kinase
PDGFRb*	18596	98	Receptor tyrosine kinase
SPARC*	20692	233	Extracellular matrix
Vimentin	22352	52	Cytoskeleton
Zeb1*	21417	13	Transcriptional regulator
Zeb2	26362	7.8	Transcriptional regulator
<i>Downregulated during EMT in vivo (*downregulation verified by immunoblot)</i>			
Gene symbol	GeneID	Fold-downregulated	Localization/function
Cdh1*	12550	43	Cell-cell adhesion
Cldn1	12737	9.8	Tight junction component
Cldn3	12739	7.6	Tight junction component
Cldn4	12740	73	Tight junction component
Cldn7	53624	44	Tight junction component
Dsg2	13867	35	Cell adhesion
ErbB2 (Her2, neu)*	13866	4.4	Receptor tyrosine kinase
ErbB3	13867	30	Receptor tyrosine kinase
Irs1*	16367	4.2	Signaling intermediate
Ocln*	18260	64	Tight junction component
Prlr	19116	27	Prolactin receptor