SUMMARY

ErbB2, a metastasis-promoting oncoprotein, is overexpressed in ~25% of invasive/metastatic breast cancers, but in 50%–60% of noninvasive ductal carcinomas in situ (DCIS). It has been puzzling how a subset of ErbB2-overexpressing DCIS develops into invasive breast cancer (IBC). We found that co-overexpression of 14-3-3ζ in ErbB2-overexpressing DCIS conferred a higher risk of progression to IBC. ErbB2 and 14-3-3ζ overexpression, respectively, increased cell migration and decreased cell adhesion, two prerequisites of tumor cell invasion. 14-3-3ζ overexpression reduced cell adhesion by activating the TGF-β/Smads pathway that led to ZFHX1B/SIP-1 upregulation, E-cadherin loss, and epithelial-mesenchymal transition. Importantly, patients whose breast tumors overexpressed both ErbB2 and 14-3-3ζ had higher rates of metastatic recurrence and death than those whose tumors overexpressed only one.

INTRODUCTION

ErbB2 overexpression is found in approximately 25% of invasive breast cancers (IBC) and is strongly associated with poor patient survival (Slamon et al., 1989). Overexpression of ErbB2 has been demonstrated to promote breast cancer invasion and metastasis (Yu and Hung, 2000). However, ErbB2 is overexpressed in 50%–60% of ductal carcinomas in situ (DCIS) in general and 60%–70% of high-grade DCIS (Nofech-Mozes et al., 2005). DCIS, a precursor of IBC, consists of clonal proliferation of malignant cells within the lumen of mammary ducts, with no evidence of invasion through the basement membrane into the surrounding stroma (Burstein et al., 2004). The apparent paradox that ErbB2, the well-known metastasis-promoting oncoprotein, is more frequently overexpressed in noninvasive DCIS than in IBC has been puzzling.

This stimulated debate about whether ErbB2 overexpression alone is sufficient to promote progression from noninvasive DCIS to IBC. The limited number of studies that have used patient follow-up data on invasive recurrence of primary DCIS have yielded ambiguous results. Some studies indicated that ErbB2-overexpressing DCIS had an increased risk of invasive recurrence (Provenzano et al., 2003), while others suggested the opposite (Perin et al., 1996; Ringberg et al., 2001). Interestingly,
The epithelial-mesenchymal transition (EMT) is a process during which epithelial cells convert to a mesenchymal cell phenotype after losing cell polarity, disassembling cell-cell adhesion machinery, and subsequently acquiring cell motility (Guarino, 2007). EMT promotes tumor invasion and metastasis by facilitating escape of tumor cells from the original rigid constraints of the surrounding tissue architecture (Guarino, 2007). The EMT-mediated increase in invasion/metastasis is largely contributed by loss of E-cadherin function because E-cadherin is essential for the maintenance of adherent junctions between neighboring cells and thus confers physical integrity on epithelial cells (Beavon, 2000; Guarino, 2007). E-cadherin loss has been shown to increase cell invasion in multiple in vitro models and has been correlated with increased metastasis in several epithelial tumor types (Strathdee, 2002). Therefore, E-cadherin is considered a suppressor of tumor invasion.

Given that ErbB2 overexpression alone in DCIS is not sufficient for progression to IBC, we explored whether 14-3-3ζ overexpression in DCIS may serve as a second hit that cooperates with ErbB2 to drive a subset of ErbB2-overexpressing DCIS progression into IBC.

RESULTS

ErbB2 and 14-3-3ζ Co-overexpression in DCIS Is Associated with Increased Invasion Potential

To investigate whether 14-3-3ζ overexpression cooperates with ErbB2 to drive a subset of ErbB2-overexpressing DCIS progression to IBC, we initially examined DCIS samples from 25 patients for whom up to 7 years of follow-up data was available. We analyzed the expression of ErbB2 and 14-3-3ζ by immunohistochemistry (IHC) staining. Fourteen of the 25 cases (56%) showed a high level of ErbB2 expression (Table 1), which is consistent with previous reports of ErbB2 overexpression in 50%-60% of DCIS cases (Nofech-Mozes et al., 2005). Eight of the 25 cases (32%) exhibited high levels of both ErbB2 and 14-3-3ζ (Table 1 and see Figure S1 available online). Strikingly, four of these eight patients had disease recurrence with distant site metastasis, suggesting that 14-3-3ζ cooperates with ErbB2 to promote the progression from DCIS to IBC and metastasis.

MCF10A, a nontransformed human MEC line, is an excellent in vitro model in 3D culture for studying breast cancer progression as it forms well-organized acinar structures that mimic the normal mammary end bud in vivo (Debnath et al., 2003). Here, we used the MCF10A 3D culture model system to study whether and how 14-3-3ζ cooperates with ErbB2 to gain invasiveness. We established multiple stable MCF10A sublines overexpressing ErbB2 (10A.ErbB2), HA-tagged 14-3-3ζ (10A.14-3-3ζ), or both ErbB2 and HA-tagged 14-3-3ζ (10A.ErbB2.14-3-3ζ), with 10A.Vec as the control (Figure 1A). We found that only the 10A.ErbB2, 10A.14-3-3ζ cells formed soft agar colonies, whereas 10A.ErbB2, 10A.14-3-3ζ, and 10A.Vec MECs did not (Figure 1B). The data indicated that ErbB2 or 14-3-3ζ overexpression alone was not sufficient to induce a full transformation in MCF10A MECs, but ErbB2 and 14-3-3ζ co-overexpression could cooperatively induce full transformation, an important step for cancer invasion/metastasis.
capacity, as many cells escaped from 10A.ErbB2.ζ acini and invaded the surrounding matrix (Figures 1C and 1D). An important feature of the noninvasive DCIS is the intact basement membrane that surrounds it, whereas invasive carcinomas are defined by loss of basement membrane integrity (Rizki and Bissell, 2004). Indeed, we observed that individual cells in 10A.ErbB2.ζ acini were patched by diffuse basement membrane protein laminin V, whereas laminin V formed a continuous basement membrane layer surrounding acini from 10A.ErbB2, 10A.14-3-3ζ, and 10A.Vec MECs (Figure 1D). Together, co-overexpression of ErbB2 and 14-3-3ζ in MCF10A MECs conferred invasiveness, whereas overexpression of ErbB2 or 14-3-3ζ alone did not.

**Invasion Is the Collective Effect of ErbB2-Mediated Increase of Cell Migration and 14-3-3ζ-Mediated Decrease of Cell-Cell Adhesion via EMT**

Tumor cell invasion is a multistep process, of which the key events include increased migration, increased protease secretion, and altered adhesion to allow dissemination from primary tumor sites (Liotta and Stetler-Stevenson, 1991). We detected no significant difference in matrix metalloproteinase levels among the four MCF10A sublines (data not shown). However, migration and wound healing assays showed that both 10A.ErbB2 and 10A.ErbB2.ζ cells had increased cell motility, whereas 10A.14-3-3ζ cells had a low motility similar to that of 10A.Vec (Figures 2A and 2B). Thus, the increased cell motility was largely contributed by ErbB2 overexpression, not by 14-3-3ζ overexpression. Multiple ErbB2 downstream signaling pathways can be involved in ErbB2-mediated cell motility, including PI3K, PAK1, Rac1, and Src activation (Feldner and Brandt, 2002). We found that Src phosphorylation is specifically increased in the two ErbB2-overexpressing MCF10A sublines compared to the two ErbB2 low-expressing MCF10A sublines (Figures S3A and S3B). Moreover, treatment with a Src kinase inhibitor (Saracatinib or AZD0530) significantly inhibited the motility of 10A.ErbB2 and 10A.ErbB2.ζ cells, whereas Rac1 and PI3K inhibitors had no significant effect (Figure S3C).

Reduced cell-cell adhesion is another prerequisite for individual cell invasion and EMT has been implicated in tumor invasion partly by reducing cell-cell adhesion ( Guarino, 2007). In contrast to 10A.Vec and 10A.ErbB2 cells that had a cobblestone-like epithelial morphology in 2D culture, 10A.ErbB2.ζ and 10A.14-3-3ζ cells displayed a spindle-like shape and exhibited a scattered distribution, indicating loss of cell-cell contact and EMT (Figure 2C). The morphological changes during EMT are
driven by a number of molecular and cellular alterations, including loss or decrease of epithelial cell markers (e.g., E-cadherin, β-catenin, α-catenin, and p120-catenin) and de novo expression of mesenchymal markers (e.g., N-cadherin, vimentin, and fibronectin). Certainly, we found that 10A.Vec and 10A.ErbB2 cells expressed high levels of E-cadherin, β-catenin, α-catenin, and p120-catenin, but minimal levels of N-cadherin and vimentin. 10A.ErbB2.ζ and 10A.14-3-3ζ cells, however, showed E-cadherin loss, dramatically reduced β-catenin, α-catenin, and p120-catenin, and de novo expression of N-cadherin and vimentin (Figures 2D and 2E). Similarly, 14-3-3ζ overexpression in HMEC-hTERT cells, immortalized by the telomerase reverse transcriptase catalytic subunit, also led to EMT (Figure S4). Thus, 14-3-3ζ overexpression contributed to the loss of cell-cell adhesion and the EMT phenotype. Together, a collective effect of ErbB2-mediated increase of cell migration and 14-3-3ζ-mediated decrease of cell-cell adhesion conferred 10A.ErbB2.ζ acini invasiveness.

Figure 2. ErbB2 Overexpression Led to Increased Cell Migration and 14-3-3ζ Overexpression Led to Decreased Cell-Cell Adhesion as a Result of EMT
(A) Transwell migration assay of the indicated MCF10A sublines. Cells that migrated to the bottom of the chamber were counted in five fields under 20x magnification. Experiments were done three times with triplicates and error bar represents SEM.
(B) Wound healing assay of the indicated MCF10A sublines. Wound closures were photographed at 0 and 6 hr after wounding. The scale bar represents 200 μm.
(C) The four MCF10A sublines exhibited different morphologies in 2D culture. The scale bar represents 100 μm.
(D) IFS of EMT markers in MCF10A sublines. E-cadherin (top, red), vimentin (bottom, red), and DAPI (blue) are shown. The scale bar represents 100 μm.
(E) Immunoblot analysis of the indicated EMT markers in MCF10A sublines. Expression of epithelial cell markers (E-cadherin, β-catenin, α-catenin, and p120 catenin) and mesenchymal cell marker (N-cadherin) were examined by immunoblot analysis in both 2D and 3D culture cell lysates.

E-Cadherin Loss, a Key Event of EMT, Is Mediated by ZFHX1B in 10A.ErbB2.ζ Cells
We next investigated how 14-3-3ζ overexpression led to E-cadherin loss, a key event of EMT resulting in decreased cell-cell adhesion. RT-PCR analysis showed that E-cadherin mRNA level was dramatically lower in 10A.ErbB2.ζ and 10A.14-3-3ζ cells than in 10A.Vec and 10A.ErbB2 cells (Figure 3A). E-cadherin mRNA loss could result from hypermethylation of its promoter (Strathdee, 2002), but we detected no significant differences in E-cadherin promoter methylation status among the four MCF10A sublines (data not shown). Another major mechanism of E-cadherin mRNA loss is direct transcriptional repression by repressors, including snail, slug, twist, E12, E47, ZFHX1B (also named SIP1), and deltaEF1 (Peinado et al., 2004). These transcriptional repressors have been found to induce EMT in vitro and their overexpression in a variety of human tumors is associated with increased tumor invasion/metastasis and poor prognosis. We examined the expression levels of snail, slug, twist, E12, E47, and deltaEF1 and found they were not significantly different among the four MCF10A sublines (Figure 3B). Interestingly, expression of ZFHX1B was dramatically higher in 10A.ErbB2.ζ and 10A.14-3-3ζ cells than in 10A.Vec and 10A.ErbB2 cells (Figure 3A). E-cadherin mRNA loss could result from hypermetylation of its promoter (Strathdee, 2002), but we detected no significant
E-cadherin loss in 10A.ErbB2,ζ and 10A.14-3-3ζ cells was due to transcriptional repression by the upregulated ZFHX1B, we cloned a fragment of E-cadherin promoter (containing two consensus ZFHX1B binding motifs: CANNTG) upstream of a luciferase reporter plasmid (pGL3.Ecad) and compared its activity among the MCF10A sublines (Figure 3D). Indeed, luciferase reporter plasmid (pGL3.Ecad) and compared its activity among the MCF10A sublines (Figure 3D). Moreover, the repression of E-cadherin promoter-driven luciferase activity was partially relieved by small interfering RNA (siRNA; Figure 3E). Therefore, ZFHX1B upregulation contributed to the transcriptional repression of E-cadherin in 10A.ErbB2,ζ and 10A.14-3-3ζ cells. In addition, examination of ZFHX1B expression in six E-cadherin-positive and four E-cadherin-negative breast cancer cell lines showed a general correlation between ZFHX1B expression and E-cadherin loss (Figure 3F).

**ZFHX1B Is Upregulated by 14-3-3ζ through Upregulation of TGF-β Receptor I and Activation of TGF-β/Smads Pathway**

Next, we investigated the mechanism of ZFHX1B upregulation in 10A.ErbB2,ζ and 10A.14-3-3ζ cells. Because TGF-β/Smads pathway activation was shown to induce EMT and was also known to be involved in ZFHX1B upregulation (Zavadil and Bottinger, 2005), we examined whether ZFHX1B upregulation by 14-3-3ζ might be due to increased TGF-β/Smads signaling. Expression of the TβRI protein, but not RNA, was dramatically increased in 10A.ErbB2,ζ and 10A.14-3-3ζ cells, whereas TβRII and 14-3-3ζ protein expression was inhibited by small interfering RNA (siRNA; Figure 3E). Therefore, ZFHX1B upregulation contributed to the transcriptional repression of E-cadherin in 10A.ErbB2,ζ and 10A.14-3-3ζ cells. In addition, examination of ZFHX1B expression in six E-cadherin-positive and four E-cadherin-negative breast cancer cell lines showed a general correlation between ZFHX1B expression and E-cadherin loss (Figure 3F).
protein levels were similar among the four MCF10A sublines (Figure 4A). Consistently, we also observed increased TjRI level in 14-3-3ζ-overexpressing HMEC-hTERT-HA-14-3-3ζ cells accompanied by upregulation of ZFHX1B (Figure S4C). The increased TjRI protein levels led to increased TGFβ-Smads activation, as indicated by the increased nuclear phospho-

ZFHX1B promoter in 10A.ErbB2. Cancer Cell 200, z activation, as indicated by the increased nuclear phospho-

increased T protein levels were similar among the four MCF10A sublines (Figure S5A).)

blocking 14-3-3ζ contributed to ZFHX1B transcriptional upregulation. Indeed, ChIP assay with the indicated antibodies showed smad3 binding with ZFHX1B promoter in 10A.ErbB2; and 10A.14-3-3ζ cells (arrows), not in 10A.Vec and 10A.ErbB2 cells (empty arrows).

(D) Knockdown of 14-3-3ζ by siRNA reduced TGFβRI and ZFHX1B expression in 10A.ErbB2; and 10A.14-3-3ζ cells. TjRI level and ZFHX1B level were analyzed by immunoblot and RT-PCR, respectively, 48 hr after siRNA transfection.

(E) 14-3-3ζ inhibited TjRI ubiquitination. 10A.ErbB2; and 10A.ErbB2 cells were cotransfected with vectors expressing myc-TjRI and HA-ubiquitin (left), 10A.ErbB2; and HeLa cells were cotransfected with myc-TjRI, HA-ubiquitin, and control/14-3-3ζ; siRNA (middle), and HeLa cells were cotransfected with myc-TjRI, HA-ubiquitin, and pcDNA3.Vec/pcDNA3.14-3-3ζ (right). After 48 hr, cell lysates were collected and subjected to immunoprecipitation and immunoblot with myc and HA antibodies.

(F) 10A.ErbB2 and 10A.ErbB2; cells were treated with DMSO or 20 μg/ml MG132 for 6 hr; TjRI levels were analyzed by immunoblot.

(G) 14-3-3ζ-associated with TjRI, 10A.ErbB2; and 10A.14-3-3ζ cell lysates were immunoprecipitated with anti-HA antibody, followed by immunoblot analysis of TjRI.

(H) Schematic representation of different TjRI mutants.

(I) 14-3-3ζ binds to TjRI at its kinase domain between amino acids 210 and 370. 10A.ErbB2; cells were infected with lentivirus expressing different TjRI mutants as in (H). Then the cell lysates were subjected to pull-down assay with GST or GST-14-3-3ζ, followed by immunoblot with TjRI antibody.
14-3-3ζ and TjiRI coexisted in the same complex (Figure 4G) and the binding region is between amino acid 210 and 370 in the kinase domain of TjiRI (Figures 4H and 4I). Immunofluorescence staining (IFS) also detected diffuse staining of both 14-3-3ζ and TjiRI proteins both in the cytosol and on the cell membrane (Figure S5B). The data are consistent with previous reports that TjiRI is constantly recycled between membrane and cellular vesicles, resulting in ~20% localization to the cell membrane and ~80% remaining in the cytosol (Di Guglielmo et al., 2003).

Most importantly, the binding of 14-3-3ζ protects TjiRI from degradation because the TjiRI-210 that cannot bind to 14-3-3ζ has a much shorter half-life compared to the TjiRI-370 that binds to 14-3-3ζ (Figure S5B). Furthermore, when 14-3-3ζ expression is knocked down by siRNA, the half-life of TjiRI is reduced by ~20% localization to the cell membrane and ~80% remaining in the cytosol (Di Guglielmo et al., 2003).

Inhibition of TGF-β/Smads pathway in 10A.ErbB2.ζ, Acini

To determine whether activation of the TGF-β/Smads pathway is required for the EMT phenotype of the 10A.ErbB2.ζ cells, we inhibited TGF-β/Smads pathway activation by treating 10A.ErbB2.ζ cells with a TGF-β receptor I/II kinase inhibitor, LY2109761 (Melisi et al., 2008). LY2109761 treatment reduced smad2/3 phosphorylation and total smad3, but had no significant effect on the phosphorylation of Akt (p-Akt) or p42-MAPK (p-P42) (Figure 5A). Interestingly, LY2109761-treated 10A.ErbB2.ζ cells adhered to neighboring cells to form cell islands, indicating improved cell-cell adhesion (Figure 5B, left). More importantly, the invasive phenotype of 10A.ErbB2.ζ cells in 3D matrigel culture was dramatically inhibited by LY2109761 treatment compared to control treatment (Figure 5B, middle). In contrast, LY2109761 treatment had no significant impact on acini development and maintenance in the other MCF10A sublines (Figure S7). Consistent with the partial reversal of EMT morphology of the cells in 2D culture and reduced invasiveness in 3D culture, there was increased epithelial protein expression, such as E-cadherin and α-catenin, after LY2109761 treatment. E-cadherin was specifically located in the membrane regions forming cell-cell contacts, a prerequisite for adherent junction formation (Figure 5B, right). Prolonged treatment also led to decreased mesenchymal protein expression (Figure 5C). Collectively, these data indicate that 14-3-3ζ-mediated TGF-β/Smads pathway activation plays a critical role in the EMT phenotype and gain of invasiveness in 10A.ErbB2.ζ cells.

Reintroduction of E-cadherin in 10A.ErbB2.ζ Cells Inhibits Invasion

Inhibition of TGF-β/Smads pathway by LY2109761 partially recovered E-cadherin expression that inhibited the invasion of 10A.ErbB2.ζ, acini, indicating that E-cadherin loss was a key event in the gain of invasiveness during EMT. To further determine the critical role of E-cadherin loss in invasion, we restored E-cadherin expression in the 10A.ErbB2.ζ cells (named 10A.ErbB2.ζ.Ecad) to levels similar to those in the 10A.14-3-3ζ cells (Figure 5D). The restored E-cadherin expression led to the recovery of other epithelial proteins, such as α-catenin, β-catenin, and p120-catenin, and reduced mesenchymal proteins, such as N-cadherin and vimentin (Figure 5E). Moreover, the cells with recovered E-cadherin expression showed a dramatic increase in cell adhesion (Figures S8A and S8B). Importantly, 10A.ErbB2.ζ.Ecad cells formed acinar structures with fewer individual cells invading into surrounding matrigel, in contrast to the highly invasive acinar structures of 10A.ErbB2.ζ.Vec cells (Figure 5F and Figure S8C). Thus, re-expression of E-cadherin in 10A.ErbB2.ζ cells efficiently increased cell-cell adhesion and inhibited, at least partially, the invasive phenotype in 3D culture. Therefore, E-cadherin loss played a critical role in inducing invasiveness of 10A.ErbB2.ζ cells.

14-3-3ζ Overexpression Is Associated with High Levels of TjiRI Expression in Both Human DCIS and IBC

We have identified TjiRI upregulation as a major mechanism of 14-3-3ζ overexpression-induced invasiveness in MCF10A. ErbB2.ζ cells. To evaluate the biological relevance of these findings, we investigated whether there is a correlation between TjiRI and 14-3-3ζ expression in patients’ samples. Because we did not have enough of the DCIS samples shown in Table 1 remaining for these staining, we stained 138 DCIS samples from patients with recently diagnosed disease and 100 IBC with clinical follow-up. We found that 14-3-3ζ overexpression significantly (p < 0.05) correlated with increased TjiRI levels in both populations (Figure 6A). Moreover, IHC staining for 14-3-3ζ, TjiRI, ErbB2, E-cadherin, vimentin, and N-cadherin on the DCIS samples showed that co-overexpression of 14-3-3ζ and TjiRI significantly (p < 0.05) correlated with (at least two) EMT marker alterations (reduced expression of epithelial marker E-cadherin and expression of mesenchymal markers vimentin and/or N-cadherin; Table S1 and Figure S9). Importantly, high 14-3-3ζ and TjiRI expression levels plus two EMT marker alterations in DCIS were significantly (p < 0.05) associated with high-grade DCIS phenotype, which correlates with a higher risk of invasive recurrence (Table S2). Representative images of multiple markers’ expressions in a pure low-grade DCIS and in a DCIS sample with microinvasion are shown in Figure 6B. Together, 14-3-3ζ overexpression in DCIS lesions correlated with TjiRI upregulation and induced EMT that could contribute to a higher risk of invasive recurrence.

Co-overexpression of ErbB2 and 14-3-3ζ Is Associated with Higher Metastatic Potential in Mice and Increased Metastatic Disease Recurrence and Death in Breast Cancer Patients

The above findings demonstrated that co-overexpression of ErbB2 and 14-3-3ζ increased the invasiveness of MECs in 3D
ErbB2 and 14-3-3ζ Cooperate in DCIS Progression

Cancer Cell

In addition, in this patient cohort, multivariate analysis demonstrated that co-overexpression of ErbB2 and 14-3-3ζ in breast tumors can predict poor prognosis (Table S3). Because a majority of these patients died of recurrent metastatic disease, these data indicated that breast cancers overexpressing both ErbB2 and 14-3-3ζ are more aggressive and have greater metastatic potential.

**DISCUSSION**

14-3-3ζ Is a Biomarker for Patients with ErbB2-Overexpressing DCIS Who Have a Higher Risk of Progression to IBC

Both clinical and experimental data support that ErbB2 overexpression plays a critical role in DCIS but is not sufficient to drive progression of the noninvasive DCIS to IBC. It has been puzzling as to what other alterations may cooperate with ErbB2 to allow a subgroup of ErbB2-overexpressing DCIS to progress to life-threatening invasive/metastatic breast tumors. Here, we identified 14-3-3ζ as a molecule that, when
co-overexpressed with ErbB2, increases the potential of DCIS to progress to IBC.

Individual tumor cell invasion is a highly complicated process that requires malignant cells to obtain at least both the “capability” (migration) and the “freedom” (dissemination) to escape from the constraint of tissue structure. We found that ErbB2 overexpression alone promoted cell migration via Src activation, but not invasion, whereas 14-3-3ζ overexpression alone had no effect on cell motility but was sufficient to reduce cell-cell adhesion. Other mechanisms, such as 14-3-3ζ loss/reduction of cell-cell adhesion, either by inducing EMT, like 14-3-3ζ, or by other mechanisms, may also promote the ErbB2-overexpressing DCIS to progress to IBC. More comprehensive investigations through unbiased analysis of both appropriate animal models and human patient samples will significantly advance our understanding of the critical step in the transition from DCIS to IBC. More importantly, for the clinical management of DCIS, evaluation of multiple proteins, including ErbB2 and 14-3-3ζ, could facilitate the identification of patients at higher risk of progressing to IBC and therefore influence the therapeutic decision.

14-3-3ζ Contributes to the Increased Invasive Ability of ErbB2-Overexpressing MECs by Inducing EMT

Accumulating evidence supports the role of EMT in promoting tumor invasion (Guarino, 2007). Pathological examination shows that malignant cells have often detached from the tumor mass at the periphery or at the invading front of the tumor. Moreover, EMT has recently been associated with “cancer stem cell” traits, suggesting a role for EMT in the initiation of recurrent tumors from disseminating cancer cells (Mani et al., 2008). However, the involvement of EMT in invasion and metastasis under a clinical setting remains controversial because of the transient and elusive nature of EMT in vivo. In this study, we detected
Clearly, further studies in larger cohorts are needed and may guide the design of strategies for intervention in the progression from noninvasive DCIS to life-threatening IBC.

EMT-mediated invasion has been largely attributed to the loss of E-cadherin, a tumor invasion suppressor (Beavon, 2000). Indeed, restoration of E-cadherin expression increased cell-cell adhesion and reduced invasion in 3D culture of the invasive 10A.ErbB2, ×1 cells (Figure 5). A key mechanism of E-cadherin loss downstream of 14-3-3ζ overexpression is ZFHX1B upregulation (Figure 3). ZFHX1B, like other E-cadherin transcriptional repressors, has been implicated in regulation of EMT during embryogenesis (Van de Putte et al., 2003), and elevated level of ZFHX1B mRNA has been reported to associate with metastasis of ovarian (Elloul et al., 2005), gastric (Rosivatz et al., 2002), and pancreatic tumors (Imamichi et al., 2007). Our findings that ZFHX1B suppressed E-cadherin in 10A.ErbB2, ×1 and 10A.14-3-3ζ cells and that high level of ZFHX1B expression correlated with E-cadherin loss in multiple breast cancer cell lines indicate a role for ZFHX1B in breast cancer cell invasion.

14-3-3ζ Overexpression Promotes TGF-β/Smads Pathway Activation

14-3-3ζ upregulated ZFHX1B by binding to TβRI and inhibiting the ubiquitin-proteasome pathway-mediated TβRI degradation, resulting in increased TβRI level, which subsequently led to TGF-β/Smads pathway activation and ZFHX1B upregulation (Figure 7B). Interestingly, overexpression of 14-3-3ζ in 293T cells has no discernable effect on ubiquitination of receptor interacting protein (data not shown), which indicates that the effect of 14-3-3ζ on TβRI ubiquitination is selective rather than an overall deregulation of the ubiquitination machinery. Furthermore, 14-3-3 protein binding can both positively and negatively regulate the stability of distinct target proteins. For example, 14-3-3ζ has been previously found to promote MDMX’s ubiquitination and degradation (LeBron et al., 2006). One possible explanation for the different effects of 14-3-3 binding is that the binding on different target proteins may either expose or mask an additional signaling motif that is essential for triggering the degradation process. Further investigation is needed to elucidate the detailed mechanism.

There are seven 14-3-3 isoforms and 14-3-3ζ can form heterodimers with other 14-3-3 isoforms. Therefore, it is possible that overexpression of other isoforms may have an effect on TβRI ubiquitination. Consistently, Schistosoma mansoni 14-3-3ζ was found to interact with SmRK1, a divergent type I TGF-β receptor, and positively regulated its signaling (Mc Gonigle et al., 2001). In contrast, despite the highly conserved sequence and tertiary structure of 14-3-3 proteins, they appear to have distinct binding specificity and affinity to various target proteins. For example, 14-3-3ζ has a unique tumor suppressor function partially by directly binding and stabilizing p53 in response to DNA damage, whereas none of other 14-3-3 isoforms share this mode of regulation (Yang et al., 2003). Therefore, further systematic studies are clearly needed to investigate the effect of other 14-3-3 isoforms on the TGF-β/Smads pathway.

The TGF-β/Smads pathway can both positively and negatively regulate tumor development (Bachman and Park, 2005). TGF-β/Smads pathway is a tumor suppressor prior to and during early tumor progression, mainly through inhibiting proliferation.

---

Figure 7. Models Represent How Co-overexpression of ErbB2 and 14-3-3ζ Cooperate in DCIS Progression

(A) A model of how co-overexpression of ErbB2 and 14-3-3ζ promotes invasion. ErbB2 overexpression increased cell proliferation and motility; 14-3-3ζ overexpression decreased cell-cell adhesion via induction of EMT. Collectively, co-overexpression of ErbB2 and 14-3-3ζ promoted cell invasion. Co-overexpression of ErbB2 and 14-3-3ζ may promote progression from DCIS to IBC via this mode of cooperation. ↑, increase; ↓, decrease; −, no significant effect.

(B) A model of 14-3-3ζ-mediated E-cadherin repression. 14-3-3ζ interacts with and stabilizes TβRI, leading to smad2/3 phosphorylation and translocation to the nucleus, where smads bind to ZFHX1B promoter to increase its transcription. ZFHX1B then represses E-cadherin transcription by binding to its promoter.

deregulation of EMT markers more frequently in DCIS overexpressing 14-3-3ζ and TβRI, which significantly associated with higher grade DCIS that had a greater risk of developing invasive recurrence. These findings strongly support the involvement of EMT in DCIS progression toward invasive/metastatic disease.
ErbB2 and 14-3-3ζ Cooperate in DCIS Progression

(Bachman and Park, 2005). Consistently, 10A.14-3-3ζ cells with increased TßRI expression proliferated at a slower rate than 10A.Vec cells (data not shown) and formed smaller acini than 10A.Vec cells. The inhibition of proliferation may result from upregulation of cell cycle inhibitors downstream of TGF-ß/Smads activation in the nontransformed MCF10A cells. In contrast, the overexpressed ErbB2 in 10A.ErbB2;ζ cells can activate various downstream signals to counter the growth-inhibitory effect of TGF-ß/Smads activation by 14-3-3ζ. However, during the later stages of tumor progression, the TGF-ß/Smads pathway can function as a tumor invasion promoter via induction of EMT (Bachman and Park, 2005). Intriguingly, 14-3-3ζ overexpression alone in MCF10A cells led to TGF-ß/Smads pathway activation and EMT (as in 10A.ErbB2;ζ cells), although without increased invasion. These data indicate that 14-3-3ζ-mediated EMT is necessary but not sufficient to promote cell invasion because of its lack of intrinsic migration ability, whereas migration is promoted by ErbB2 overexpression in 10A.ErbB2;ζ cells that become invasive. Our findings are consistent with a previous report that ErbB2 activation can cooperate with TGF-ß treatment to promote invasion (Seton-Rogers et al., 2004). Conversely, bitransgenic mice that expressed MMTV-neu and a soluble antagonist of TGF-ß had a significant reduction of metastasis (Yang et al., 2002). Our findings on the synergistic effect of ErbB2 overexpression and 14-3-3ζ-mediated activation of TGF-ß/Smads pathway shed light on molecular mechanisms of gain of invasiveness during ErbB2-overexpressing DCIS progression, which is contributed by ErbB2-induced motility and proliferation plus 14-3-3ζ-mediated loss of cell-cell adhesion via inducing EMT. Recently, the TGF-ß/Smads pathway was implicated to play a critical role in the “communication” of MECs with their “natural invasion suppressors,” myoepithelial cells (Hü et al., 2008). The impact of ErbB2 and 14-3-3ζ co-overexpression on myoepithelial cells will be investigated in future studies.

Our findings that ErbB2 and 14-3-3ζ co-overexpression in DCIS predicts a higher risk of progression to IBC also provide molecular targets for designing combination therapies to intervene in DCIS progression. Targeting 14-3-3ζ may be challenging at the current stage because 14-3-3ζ regulates many important proteins that are essential for homeostasis. Identification of the TGF-ß/Smads pathway as a downstream event of 14-3-3ζ overexpression in promoting invasion represents an opportunity for therapeutic intervention. Currently, the TGF-ß/Smads pathway is under intensive investigation as a therapeutic target (Dumont and Arteaga, 2003; Yingling et al., 2004). Given the dichotomous role of the TGF-ß/Smads pathway in tumor development, it is critical to dissect the TGF-ß/Smads downstream signals and their crosstalk with other signaling networks, such as ErbB2 signaling, in order to specifically activate its tumor-suppressing role or specifically inhibit its tumor-promoting role. Our findings suggest the potential therapeutic benefit of inhibiting the TGF-ß/Smads pathway in the context of ErbB2 and 14-3-3ζ co-overexpressing breast cancers.

**EXPERIMENTAL PROCEDURES**

**Tissue Specimens**

Twenty-five DCIS and 107 IBC specimens were obtained from the Cancer Hospital, FuDan University (Shanghai, China). An additional 140 DCIS were collected at The University of Texas M.D. Anderson Cancer Center (MDACC; Houston, TX). Patient samples were collected and processed in compliance with protocols approved by the MDACC Institutional Review Board and by the Cancer Hospital/Cancer Institute, FuDan University Institutional Review Board. Detailed clinic-pathological information of these patient cohorts are provided in Tables S4–S6.

**Cells, Constructs, Antibodies, and Reagents**

The MCF10A cell line was a kind gift from Dr. Robert Pauley (Karmanos Cancer Institute, Detroit, MI) and was cultured in 3D culture as previously described (Debnath et al., 2003). The HMEC-HRTERT cell line was kindly provided by Dr. Victoria Seewaldt (Duke University, Durham, NC). ErbB2, HA-14-3-3ζ, and E-cadherin genes were cloned into pLPXC, pLN CX2, and pLH CX vectors (Clontech), respectively. Retroviral infection was done as previously described (Danes et al., 2008). Stable clones were selected with 400 μg/ml neomycin, 800 ng/ml puromycin, and 100 μg/ml hygromycin, respectively. Multiple stable clones were used to rule out potential clonal effects. HA-14-3-3ζ was also cloned into pRc/3 (with GFP in the backbone) and pLOVE lentiviral vectors. Lentivirus production and infection, and reagents and antibodies used, are described in the Supplemental Experimental Procedures.

**Soft Agar Colony Formation Assay, Cytoplasm and Nuclear Protein Fractionation, Immunoblotting, IHC, IFS, Immunoprecipitation, RNA Extraction, RT-PCR, and Quantitative RT-PCR, Luciferase Reporter Assay, Migration Assay, Wound Healing Assay, Cell Adhesion Assay, and Cell Aggregation Assay**

The detailed procedures are described in the Supplemental Experimental Procedures. IFS analysis of monolayer cell cultures was done as previously described (Debnath et al., 2003). For IFS of 3D cultures, acini were embedded in sucrose and frozen in Tissue-Tek OCT (Sakura Finetek), and 5 μm frozen sections were cut and subjected to analysis (Weaver et al., 1997).

**siRNA Transfection and ChIP**

ON-TARGET plus SMART Pool siRNA for 14-3-3ζ, ON-TARGET plus control siRNA and ZFHX1B siRNA were purchased from Dharmacon. Transfection was done as previously described (Danes et al., 2008). ChIP assay was performed with ChIP-IT kit from Active Motif according to the manufacturer’s instructions. The DNA pulled down by antibodies was amplified with ZFHX1B promoter-specific primers.

**Cell Adhesion Assay and Cell Aggregation Assay**

For the cell adhesion assay, 96-well plate was coated with fibronectin (10 μg/ml) before use. Ten thousand cells were resuspended as single-cell suspension in 200 μl DMEM/F12 media containing 0.5% BSA and added to the coated plate for incubation at 37°C for 1 hr. Nonadherent cells were washed away with DMEM/F12 media containing 0.1% BSA. Adherent cells were detected by MTS assay according to the manufacturer’s instruction. For aggregation assay, a six-well plate was coated with poly 2-hydroxyethyl methacrylate (polyHEMA) before use. Fifty thousand cells were resuspended to single cell suspension in 500 μl DMEM/F12 containing 0.5% BSA, added to the polyHEMA-coated plates, and incubated at 37°C on a rotating platform (150 rpm) for 30 min. Cells were then fixed with methanol and photographed under a phase-contrast microscope.

**Statistical Analysis**

Statistical tests used to analyze data included Fisher’s exact test, log-rank test, Chi-square test, and Student’s t test. Multivariate statistical analysis was performed using a Cox regression model. Statistical analysis was performed using SPSS for Windows (16.0; SPSS, Inc.) and GraphPad Prism (Prism 5.0; GraphPad Software Inc.) packages. A p value < 0.05 was considered significant.

**SUPPLEMENTAL DATA**

Supplemental Data include ten figures, six tables, and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/cancer-cell/supplemental/S1535-6108(09)00256-6.
ACKNOWLEDGMENTS

We thank J.M. Shu, Dr. J.P. Issa, and Dr. X. Lin (MDACC), and Dr. Y. Higashi (Osaka University) for their technical support and ZFHXB1 antisemur. D.Y. is the Nylen Eckles Distinguished Professor in Breast Cancer Research at MDACC. W.T. is partially supported by the Royal Golden Jubilee Program, Thailand Research Fund. This work is supported by National Institutes of Health grants P09-CA 16672 (MDACC), R01-CA109570, R01-CA112567, P01-CA099031 project 4, and P50 CA116199 project 4, Department of Defense Center of Excellence grant subproject W81XWH-06-2-0033 and Synergistic Award W81XWH-08-1-0712; and Susan G. Komen Breast Cancer Foundation Promise Grant KG091020 (D.Y.).

Received: October 16, 2008
Revised: May 20, 2009
Accepted: August 11, 2009
Published: September 8, 2009

REFERENCES


