

Bcl-2-Mediated Cell Survival Promotes Metastasis of EpH4 β MEKDD Mammary Epithelial Cells

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Abstract

The majority of patients who succumb to cancer die from metastatic disease progression rather than from the primary tumor. Elucidation of the mechanisms underlying tissue-specific metastasis is essential to the development of effective therapies. The mitogen-activated protein kinase kinase (MEK) pathway is frequently activated in human tumors and has been shown to regulate genes involved in proliferation, migration, and invasion. Studies with MEK-transformed EpH4 mouse mammary epithelial cells showed that these cells are highly tumorigenic but have a limited metastatic ability. Detachment of epithelial cells from the extracellular matrix causes disruption of the actin cytoskeleton and induces apoptosis. Several metastatic breast carcinoma cell lines have been shown to be resistant to cell death following actin disruption. This death-resistant phenotype can be modeled by overexpressing the antiapoptotic Bcl-2 protein in cells. This suggests that mechanisms that regulate survival of extravasated tumor cells may enhance metastatic efficiency. Therefore, we examined whether expression of Bcl-2 in MEK-transformed EpH4 mammary epithelial cells could provide a survival advantage and promote metastasis. Expression of Bcl-2 in parental EpH4 mammary epithelial cells or MEK-transformed cells was insufficient to induce increased migration, invasion, or tumor development. However, Bcl-2 expression markedly enhanced spontaneous lung metastasis from orthotopically implanted primary tumors. These results clearly show that mechanisms that regulate primary tumor development are distinct from those that promote metastasis and that assays designed to isolate genes involved in transformation may fail to identify genes that are critical regulators of metastasis. (Mol Cancer Res 2004;2(10):551–6)

Introduction

The process of metastasis is highly complex and involves dissemination of cells from the primary tumor through the lymphatic and vascular systems coupled with a tissue-selective

ability to colonize distant tissues and organs (1). Mechanisms controlling cellular proliferation, migration, and invasiveness have been identified as key regulators of primary tumor development, but it is unclear whether these are the critical mechanisms that control metastasis. The identification of genes regulating breast cancer metastasis has been the subject of intense investigation (2). A recent study using the MDA-MB-231 human breast carcinoma cell xenotransplant model identified a multigene expression program that regulated bone-specific metastasis (3). These data suggest that genes that control metastasis may play a limited role in primary tumor development.

Constitutive activation of the mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK pathway is a frequent event in tumors derived from the breast, colon kidney, and lung (4-7). Hyperactivation of the MEK signal transduction pathway is a frequent event in human breast cancer (4) that correlates strongly with increased lymph node involvement and metastasis (7). However, the molecular targets and outcomes of MEK-MAPK signal transduction are highly cell specific and tumor type specific. Transfection of constitutively active MEK1 induced matrix metalloproteinase expression (8) and mediated transformation of fibroblast (8, 9) and epithelial (10, 11) cell lines, but it is unclear which activities downstream of MEK-MAPK signal transduction are required to promote spontaneous metastasis. We have chosen to use the mammary fat pad transplantation model to dissect mechanisms regulating primary tumor development and spontaneous metastasis in immune-competent mice (12). Previous studies showed that MEK-transformed EpH4 mouse mammary epithelial cells were highly invasive *in vitro* and tumorigenic but showed limited metastatic ability (11). One of the critical steps in the metastatic cascade is the detachment of cells from the primary tumor and their subsequent dissemination through the vascular system. Detachment of epithelial cells from the extracellular matrix has been shown to induce apoptosis (13), and it has been proposed that suppression of apoptosis could promote primary tumor development and metastasis. Apoptosis from reduced extracellular matrix attachment may be mediated by the resulting disorganization of the cytoskeleton (14). Disruption of the actin cytoskeleton in human mammary epithelial cells by latrunculin-A, a monomeric G-actin binding compound that inhibits actin polymerization, has been shown to potently induce apoptosis, which could be rescued by Bcl-2 (15). In addition to its well-described antiapoptotic role, expression of Bcl-2 in a rat glioma cell line resulted in the induction of matrix metalloproteinase activity (16). Therefore, the effect of Bcl-2 to enhance primary tumor development and metastasis of EpH4 parental and MEK-transformed cells was determined. The results clearly show that Bcl-2 enhanced

Received 7/16/04; revised 8/31/04; accepted 9/1/04.

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the metastatic capacity of MEK-transformed EpH4 cells without affecting cell motility and invasiveness or primary tumor growth. This indicates that distinct mechanisms regulate primary tumor development and metastasis.

Results

To address the role of the cell survival protein Bcl-2 in breast tumorigenesis and metastasis, we used the EpH4 mouse mammary epithelial cell line (17) and MEK-transformed EpH4 cell lines that had been shown previously to be highly tumorigenic when transplanted into cleared mammary fat pads (11). Full-length human Bcl-2 was stably expressed in EpH4 parental and MEK expressing cell lines, and Western blot analysis identified multiple clones expressing high levels of Bcl-2 as compared with parental EpH4 cells (Fig. 1A). Equivalent protein loading was determined by probing for β -actin expression (Fig. 1B). The Bcl-2 expressing clones were morphologically indistinguishable from the parental EpH4 and MEK expressing cell lines, but the Bcl-2 expressing clones did show slightly reduced rates of proliferation *in vitro* (data not shown). The reduced rate of proliferation is most probably due to the growth inhibitory effect of full-length Bcl-2 that is independent of its antiapoptotic effect (18).

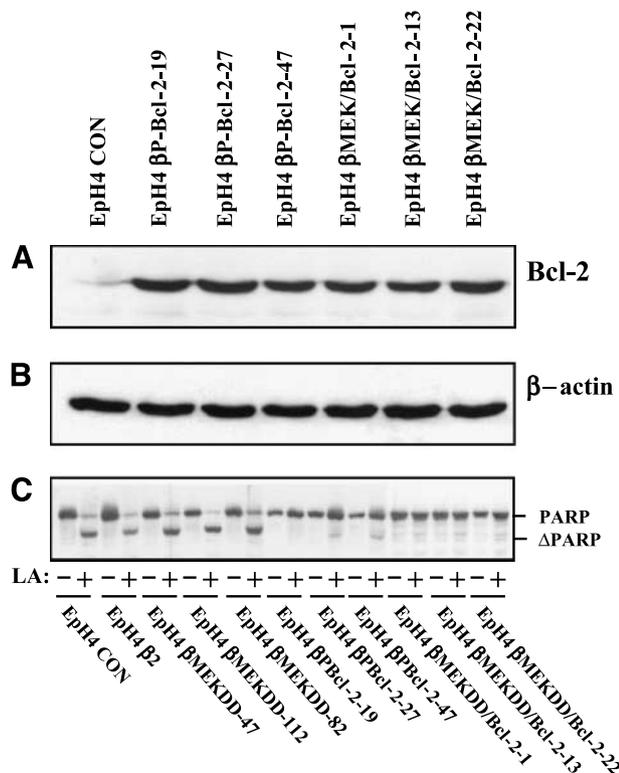


FIGURE 1. Bcl-2 overexpression inhibits apoptosis induced by disruption of the actin cytoskeleton. Western blot analysis of (A) Bcl-2 and (B) β -actin protein levels in EpH4 parental and β MEKDD cell lines. C. Parental EpH4 cells (CON) or stable lines expressing empty vector (β 2), MEK, Bcl-2, or both MEK and Bcl-2 (as indicated) were treated with vehicle (0.1% ethanol) or latrunculin-A (2.5 mmol/L) for 28 hours. Cell lysates were subjected to SDS-PAGE and Western blotting using a polyclonal anti-PARP antibody, which recognizes both full-length PARP and the caspase-cleaved form (Δ PARP).

Disruption of actin polymerization in MCF10A human mammary epithelial cells using latrunculin-A has been shown previously to induce apoptosis through a poly(ADP-ribose) polymerase (PARP)-dependent mechanism that could be rescued by Bcl-2 (15). Loss of cytoskeletal organization is thought to signal cell death in response to reduced attachment to the extracellular matrix by inducing loss of Akt phosphorylation and reducing Bcl-2 expression. To determine whether disruption of the actin cytoskeleton induced apoptosis in mouse mammary epithelial cells, EpH4 parental and MEK expressing cells were treated with latrunculin-A for 28 hours, and the cleavage of PARP was determined to measure apoptotic commitment (Fig. 1C). EpH4 parental and MEK-transformed cells were equally sensitive to latrunculin-A-induced cytoskeletal disruption and underwent apoptosis as determined by nearly complete cleavage of PARP. Expression of Bcl-2 efficiently rescued both parental EpH4 and MEK-transformed cells from latrunculin-A-induced apoptosis (Fig. 1C).

Matrigel-coated Boyden chambers represent one of the most frequently used *in vitro* models of invasiveness. This assay mimics the early steps of tumor invasion *in vivo* by requiring proteolytic degradation of the basement membrane before cells can gain access to the pores in the filter inserts. Studies examining the ability of Bcl-2 to enhance migration and invasion have been mixed depending on the cell line examined (19, 20), suggesting that this represents an indirect effect of Bcl-2 expression. Modified Boyden chamber assays were done to quantify the migratory and invasive capacity of the Bcl-2 expressing EpH4 cell lines. MEK-transformed EpH4 cells have been shown previously to have increased migratory and invasive capacity as compared with parental EpH4 cells (11), and the ability of Bcl-2 to enhance this activity was examined (Fig. 2). These results clearly show that expression of Bcl-2 was not sufficient to enhance the *in vitro* migration or invasion rates of parental EpH4 or MEK-transformed cells.

The effect of Bcl-2 expression in EpH4 cell lines on primary tumor development and metastasis *in vivo* was determined using the mammary transplantation model (12). EpH4 parental, β PBcl-2, and β MEKDD/Bcl-2 cells were transplanted (5×10^5 cells/10 μ L) into cleared number 4 mammary fat pads of syngeneic BALB/c hosts, and the animals were monitored for tumor development. Mammary fat pads transplanted with EpH4 β PBcl-2 cell lines did not form tumors over the course of the experiment (4-5 months; Table 1), and these results are comparable with previous studies with parental EpH4 cells (11). However, EpH4 β MEKDD/Bcl-2 cells rapidly formed mammary tumors of 1 to 2 cm^3 by 5 to 7 weeks (Table 1). Comparison of EpH4 β MEKDD (11) and β MEKDD/Bcl-2 outgrowths showed that the kinetics of primary tumor development were indistinguishable, and both sets of clones formed poorly differentiated mammary carcinomas characterized by intense stromal reactivity (data not shown).

The spectrum and kinetics of metastasis were measured by detailed histopathologic analyses of internal organs and tissues from tumor-bearing animals for macroscopic metastatic lesions. Previous studies with animals bearing EpH4 β MEKDD tumors

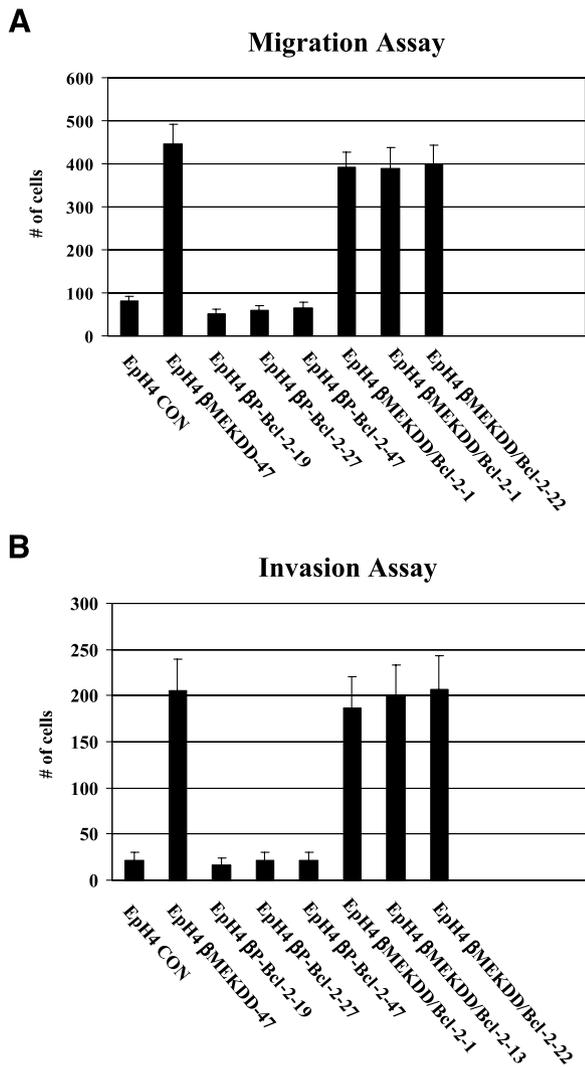


FIGURE 2. Expression of Bcl-2 does not enhance migratory and invasive potential of EpH4 cells expressing constitutively activated MEK1 in modified Boyden chamber assays *in vitro*. EpH4 parental (CON), β MEKDD-47, and three independent β Bcl-2 and β MEKDD/Bcl-2 cell lines that had (A) migrated through uncoated filter inserts or (B) invaded through Matrigel-coated filter inserts were fixed, stained, and counted. Each experiment was done in triplicate. Columns, average; bars, SD.

showed that metastatic cells were readily reisolated from several internal organs such as lung and spleen (11), but macroscopic lesions that represented tumor cells, which had extravasated and invaded into the adjoining parenchyma, were difficult to identify (Table 1; Fig. 3A). However, animals bearing β MEKDD/Bcl-2 mammary tumors presented with numerous macroscopic metastatic lesions that had invaded into surrounding lung tissue, and the difference in the metastatic burden between the groups was dramatic (Table 1; Fig. 3). The increase in metastasis in animals bearing EpH4 β MEKDD/Bcl-2 mammary tumors seemed to be specific to the lung, because no increase in metastasis was seen in any other tissues (data not shown). These data clearly show that cell survival factors such as Bcl-2, which do not accelerate primary tumor development, can dramatically enhance metastasis.

Discussion

The presence of metastasis-promoting genes is the subject of intense debate, and several recent studies argue that the metastatic potential of a primary human tumor can be determined by its gene expression signature (21, 22). It is unclear whether these gene expression signatures that are predictive of disease recurrence and metastasis correspond to clonal variants within the primary tumor that have enhanced metastatic ability or to the primary tumor as a whole. Karyotypic and genomic analyses of breast carcinomas showed that metastases frequently shared common features of the primary tumor from the same patient, supporting the clonal progression model (23, 24). However, a recent report challenges this paradigm and presents evidence that tumor cells disseminate early and develop metastatic lesions independent from the primary tumor (25, 26). In either case, these studies support the contention that genomic changes in cells in the primary tumor, which probably result in changes in gene expression, can effect metastatic progression.

Several recent studies have showed that modulation of gene expression can alter the metastatic potential of tumor cells. A recent study elucidated a multigenic expression program that regulates bone-specific metastasis of MDA-MB-231 human breast carcinoma cells (3). Interestingly, many of the genes identified code for osteolytic factors and cytokines that are not predicted to promote primary tumor growth but should enhance the ability of disseminated tumor cells to thrive in the bone microenvironment (3). Gene expression analysis in a murine model of osteosarcoma identified the cytoskeletal protein Ezrin as being strongly associated with metastasis (27). Further functional studies showed that Ezrin expression provided an essential survival signal for tumor cells that allowed their establishment of metastases in the lung (28). These studies reveal the importance of survival signals in enhancing metastatic progression of osteosarcoma.

Metastatic human breast carcinoma cell lines were shown to be resistant to cell death induced by disruption of the actin cytoskeleton *in vitro*, and this phenotype could be reproduced in nontumorigenic cell lines by overexpression of Bcl-2 (15). Based on these results, an unbiased cytoskeleton-based functional genetic screen identified the antiapoptotic Bcl-x_L protein as an enhancer of metastasis independent of any effects on primary

Table 1. Tumor Development in EpH4 Mammary Fat Pad Transplants Lung

Clone	Outgrowth*	Tumor	Metastasis†
EpH4 β P-Bcl-2-19	13/16	0/16	0
EpH4 β P-Bcl-2-27	12/14	0/14	0
EpH4 β P-Bcl-2-47	18/22	0/22	0
EpH4 β MEKDD-47	22/22	22/22	0
EpH4 β MEKDD-112	16/16	16/16	0
EpH4 β MEKDD-116	16/16	16/16	0
EpH4 β MEKDD/Bcl-2-1	18/18	18/18	5 \pm 3
EpH4 β MEKDD/Bcl-2-13	16/16	16/16	7 \pm 5
EpH4 β MEKDD/Bcl-2-22	20/20	20/20	12 \pm 6

*Outgrowth measured by whole mount analysis or by H&E staining of formalin-fixed transplanted mammary fat pads.

†Metastasis scored by counting macroscopic metastatic lesions using a dissecting microscope. Values represent average \pm SD.

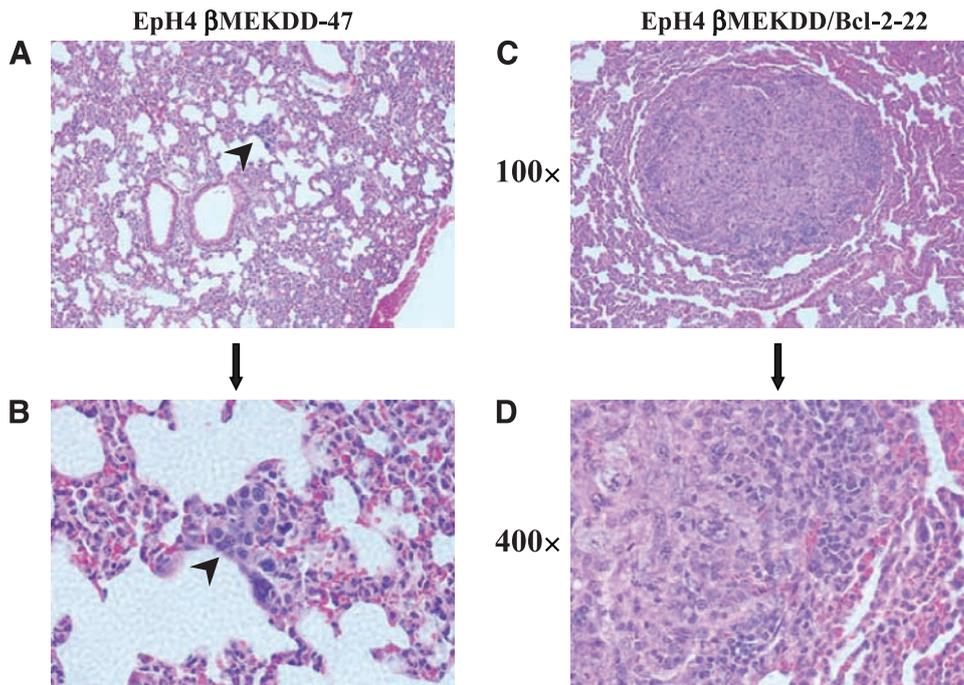


FIGURE 3. Increased spontaneous lung metastasis in EpH4 β MEKDD/Bcl-2 mammary transplants. H&E-stained sections of (A and B) EpH4 β MEKDD-47 and (C and D) EpH4 β MEK/Bcl-2-22 lung metastases. A and B. Arrowhead, the largest lung metastasis seen in EpH4 β MEKDD mammary transplants.

tumor development (29). In support of this finding are previous studies demonstrating that overexpression of Bcl-2 and Bcl-x_L in metastatic human breast carcinoma cell lines can enhance survival of disseminated tumor cells in the vasculature of immune-deficient animals bearing s.c. xenografts (30, 31).

The mechanisms regulating metastasis are complex and still poorly understood, and evidence is increasing that genes involved in metastasis may not be identified in standard transformation assays. The data presented here show that Bcl-2 expression had no effect on *in vitro* proliferation and invasion assays but that it markedly enhanced spontaneous lung metastasis from orthotopically implanted primary tumors. These results clearly show that mechanisms controlling metastasis can be regulated independently from primary tumor development and suggest an important role for antiapoptotic proteins such as Bcl-2 in enhancing pulmonary metastasis in human patients.

Materials and Methods

Cell Culture

Eph4 mouse mammary epithelial cells derived from BALB/c mice were obtained from Hartmut Beug of the Research Institute of Molecular Pathology (Vienna, Austria). Cells were cultured in monolayer on tissue culture plastic (Corning, Corning, NY) in DMEM supplemented with 10% bovine calf serum, penicillin-streptomycin (100 μ g/mL each), and L-glutamine (2 mmol/L). Latrunculin-A (Biomol, Plymouth Meeting, PA) was reconstituted as a 5 mmol/L solution in ethanol and stored at -20°C and all experiments with latrunculin-A were done in serum-free DMEM.

Vectors and Transfections

To target high-level expression in EpH4 β MEKDD cells, a new expression construct was created. The pCAGGS vector containing the cytomegalovirus IE enhancer/chicken β -actin promoter (32) was modified by inserting a SV40-Puromycin cassette from pPur (Clontech, Palo Alto, CA) and expanding the multiple cloning site by ligating an oligonucleotide linker containing *Xho*I, *Kpn*I, *Hind*III, *Eco*RI, *Bam*HI, *Bgl*II, *Not*I, and *Cla*I restriction sites to create p β P. The new vector (p β P) uses the strong chicken β -actin promoter to drive transgene expression, contains an extended multiple cloning site, and has a puromycin resistance cassette for selection in eukaryotic cells. Full-length human Bcl-2 was subcloned from pCDNA3-Bcl-2 (15) into p β P and then transfected into EpH4 cells using Fugene-6 (Roche, Indianapolis, IN). After selection in complete medium supplemented with 2 μ g/mL puromycin (Sigma Chemical Co. St. Louis, MO) for 14 days, individual clones were isolated and expanded.

Western Blotting

Eph4 cells grown in complete medium or treated in serum-free DMEM containing 0.1% ethanol or latrunculin-A were washed with PBS and then lysed in 150 mmol/L NaCl, 50 mmol/L Tris (pH 7.6), 10 mmol/L sodium pyrophosphate, 10 mmol/L NaF, 2 mmol/L EDTA, 1% NP40, 0.1% SDS, 0.5% deoxycholate, 1 mmol/L sodium orthovanadate, 10 mmol/L β -glycerophosphate, and Complete protease inhibitor tablets (Roche). Protein concentration in the lysates was measured using Bradford reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. Equivalent amounts of protein were separated by SDS-PAGE on 12% polyacrylamide gels and then

transferred to Immobilon-P membranes (Millipore, Bedford, MA) using a SemiPhor transfer apparatus (Hoeffer, Piscataway, NJ). Western blotting was done according to the manufacturer's protocols with the following antibodies: polyclonal anti-PARP (H-250, Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti- β -actin (AC-15, Sigma Chemical), and monoclonal anti-Bcl-2 (Transduction Laboratories, Lexington, KY). Proteins were visualized using a horseradish peroxidase-linked secondary antibody (Jackson ImmunoResearch, West Grove, PA) and a chemiluminescent detection system (enhanced chemiluminescence, Amersham, Sunnyvale, CA).

Boyden Chamber Assays

Migration and invasion assays were done using modified Boyden chambers with filter inserts for 24-well dishes containing 8 μ m pores (Becton Dickinson, Franklin Lakes, NJ) as described previously (11, 33). Uncoated filters were used for migration assays, and Matrigel-coated filters were used for invasion assays. Cells (1×10^5) were plated into 300 μ L of serum-free mammary epithelial growth medium in the upper chamber, and the lower chamber was filled with 500 μ L of mammary epithelial growth medium (Clonetics, San Diego, CA). After 24 hours in culture, cells were fixed in 2.5% glutaraldehyde in PBS for 5 minutes and then stained with 0.5% toluidine blue in 2% Na_2CO_3 for 5 minutes. Cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed in PBS. Cells on the underside of the filters were counted by microscopic inspection. Each clone was plated in triplicate per experiment, and each experiment was repeated twice.

Mammary Gland Transplantation

BALB/c mice were obtained from Charles River Labs (Wilmington, MA) and were maintained according to the Institutional Animal Care and Use Committee-approved guidelines. EpH4 cells were transplanted into the cleared number 4 mammary fat pads of 21- to 25-day-old female syngeneic BALB/c mice by standard procedures (11, 34). Briefly, EpH4 cells were grown to confluence, harvested, and resuspended in PBS; then, 5×10^5 cells/10 μ L were injected into both cleared number 4 mammary fat pads distally to the lymph node using a Hamilton syringe. Starting at 1 week after transplantation, the mice were palpated biweekly for mammary tumors. EpH4 β MEKDD and β MEKDD/ β PBcl-2 transplants gave rise to palpable mammary tumors within 7 to 10 days, and the animals were sacrificed 5 to 7 weeks post-transplant with mammary tumors 1 to 2 cm^3 . EpH4 control and β PBcl-2 transplanted mammary fat pads were either isolated at 8 weeks post-transplant to assess outgrowth or observed for >6 months to monitor long-term tumor development. Tissues were fixed in 10% neutral-buffered formalin (Fisher, Pittsburgh, PA) and were processed for routine H&E staining and histopathology.

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Molecular Cancer Research

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Mol Cancer Res 2004;2:551-556.

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