Extracellular Matrix Metalloproteinase Inducer (CD147) Confers Resistance of Breast Cancer Cells to Anoikis through Inhibition of Bim*

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Overexpression of extracellular matrix metalloproteinase inducer (EMMPRIN or CD147), a member of the immunoglobulin family and a glycoprotein enriched on the surface of tumor cells, promotes invasion, metastasis, and growth and survival of malignant cells and confers resistance to some chemotherapeutic drugs. However, the molecular mechanisms underlying the actions of EMMPRIN are not fully understood. In this study we sought to determine whether EMMPRIN contributes to the malignant phenotype of breast cancer by inhibiting anoikis, a form of apoptosis induced by loss or alteration of cell-cell or cell-matrix anchorage, and to explore the signaling pathways involved. We found that in the absence of attachment, human breast carcinoma cells expressing high levels of EMMPRIN formed less compact aggregates with larger surface area and less fibronectin matrix assembly, had higher viability, and were resistant to anoikis. Knockdown of EMMPRIN expression by RNA interference (small interfering RNA or short hairpin RNA) sensitized cancer cells to anoikis, as demonstrated by activation of caspase-3, increased DNA fragmentation, and decreased cellular viability. Furthermore, we observed that the accumulation of Bim, a proapoptotic BH3-only protein, was reduced in EMMPRIN-expressing cells and that silencing of EMMPRIN expression elevated Bim protein levels and enhanced cellular sensitivity to anoikis. Treatment of cells with a MEK inhibitor (U0126) or proteasome inhibitor (epoxomicin) also up-regulated Bim accumulation and rendered cells more sensitive to anoikis. These results indicated that expression of EMMPRIN protects cancer cells from anoikis and that this effect is mediated at least in part by a MAP kinase-dependent reduction of Bim. Because anoikis deficiency is a key feature of neoplastic transformation and invasive growth of epithelial cancer cells, our study on the role of EMMPRIN in anoikis resistance and the mechanism involved underscores the potential of EMMPRIN expression as a prognostic marker and novel target for cancer therapy.

EMMPRIN3 (also known as CD147, basigin, M6, and tumor cell-derived collagenase stimulatory factor) is a glycoprotein that belongs to the immunoglobulin superfamily and is enriched on the plasma membrane of many human cancer cells. These cancers include breast cancer, lymphoma, oral squamous cell carcinoma, glioma, melanoma, lung, and bladder and kidney carcinomas (1). The known functions of EMMPRIN include stimulating the production of multiple matrix metalloproteinases by fibroblasts, endothelial cells, and tumor cells (2), interacting with certain lactate transporters (MCT1 and MCT4) and facilitating their expression on the cell surface (3), regulating store-operated calcium entry (4), and serving as a receptor for extracellular cyclophilins (5).

The expression of EMMPRIN plays an important role in tumor formation and invasion/metastasis in both animal models (6) and cancer patients (7). Human breast cancer cells transfected with an EMMPRIN expression vector showed increased tumorigenicity and invasive potential in mice (6). In human cancers, the expression of EMMPRIN correlated with invasion and metastasis (8–10). In recently reported clinical studies, high levels of EMMPRIN were found on ~90% of micrometastatic cells in the bone marrow of breast cancer patients (7), and expression of EMMPRIN was associated with poor prognosis, higher tumor grade, increased tumor size, and a higher mitotic index (7). Also, significantly higher expression of EMMPRIN was found in malignant effusions than in primary tumors in patients with breast cancer (11). Our laboratory previously identified the up-regulation of EMMPRIN in multidrug-resistant cancers cells and its role in stimulating several matrix metalloproteinases (12). Misra et al. (13) recently demonstrated that EMMPRIN is involved in the resistance of cancer cells to a variety of chemotherapeutic agents. Marieb et al. (14) found that EMMPRIN promotes anchorage-independent growth of human breast carcinoma cells in a hyaluronan-dependent manner. These studies suggest a role of EMMPRIN in regulating survival of cancer cells, which may favor their growth and dissemination and resistance to treatment. Yet, the mechanism underlying the survival-promoting function of EMMPRIN remains unclear. In the present study, we found that expression of EMMPRIN in human breast carcinoma cells rendered them resistant to anoikis, a form of apoptosis triggered by a lack of or improper cell-matrix interactions. We also demonstrated that the suppression of anoikis by EMMPRIN was mediated by down-regulation of the pro-

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3 The abbreviations used are: EMMPRIN, extracellular matrix metalloproteinase inducer; shRNA, short hairpin RNA; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MEK, MAP/ERK kinase; poly-HEMA, poly(2-hydroxyethyl methacrylate); TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.
apoptotic BH3-only protein, Bim, through a MAP kinase-dependent pathway.

**MATERIALS AND METHODS**

**Cell Lines and Culture**—EMMPRIN sense- and antisense-transfected MDA-MB-231 human breast cancer cell lines were generated and cultured as previously described (15). Human breast cancer cells, MDA-MB-436, transfected with an EMMPRIN expression vector or empty vector (MDA-MB-436/EMMPRIN and MDA-MB-436/V) as described previously (6), were generous gifts from Dr. Stanley Zuker (Veterans Affairs Medical Center, Northport, NY). These cell lines were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂, 95% air. All cultures were monitored routinely and found to be free of contamination by mycoplasma or fungi. All cell lines were discarded after 3 months, and new lines were propagated from frozen stocks.

**Antibodies and Reagents**—The antibodies used in this study were purchased from the following sources: monoclonal anti-EMMPRIN antibody, Research Diagnostics, Inc. (Flanders, NJ); fluorescein isothiocyanate-conjugated mouse anti-EMMPRIN antibody, BD Pharmingen; polyclonal anti-p44/42 MAP kinase, anti-phospho-p44/42 MAP kinase antibodies, and polyclonal cleaved caspase-3 antibody, Cell Signaling Technology, Inc. (Beverly, MA); polyclonal caspase-3 antibody recognizing the full-length proenzyme, Biomol (Plymouth Meeting, PA); polyclonal anti-Bim antibody, BD Biosciences; polyclonal anti-fibronectin antibody ab6584, AbCam, Ltd. (Cambridge, UK); monoclonal anti-β-actin antibody, Sigma; MEK inhibitor U0126, Promega; proteasome inhibitor epoxomicin, Calbiochem; Bim-siRNA, Dharama RNA Technologies (Lafayette, CO).

**siRNA, shRNA Expression Vectors, and Transfection**—The siRNA sequence and DNA hairpin oligonucleotides corresponding to the cDNA sequence of EMMPRIN (accession number NM_001728) were designed with reference to the Ambion (Austin, TX) and Dharama design centers using the Tuschl rules (16). The siRNA duplex with the following sense and antisense sequences was used: 5'-GUACAAGAUCUGACUCUUU (sense) and 5'-AGAGUCAGUGAUCUUGUACUU (antisense). The DNA hairpin oligonucleotides were synthesized using Ambion’s shRNA design center and their default loop sequence. Oligonucleotides were cloned into Ambion’s pSilencer 2.1 vectors according to the manufacturer’s instructions. Large scale preparations of the vectors were made using HiSpeed Plasmid Maxi Kit (Qiagen, Valencia, CA) and sequenced to confirm the presence of the correct shRNA sequence. pSilencer vectors were linearized with the AflIII restriction endonuclease prior to transfection.

The day before transfection, MDA-MB-231 cells (3 x 10⁶ cells/dish) were seeded into 100-mm² dishes. siRNA, the shRNA expression vector, or control vector was transfected into the cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. To select the cells expressing shRNA, cells were harvested and reseeded into 100-mm² dishes at 10- and 100-fold dilutions in Dulbecco’s modified Eagle’s medium containing hygromycin B (250 mg/ml). Clones were grown under antibiotic selection, expanded to 6-well plates, and then screened for EMMPRIN expression by staining with a fluorescein reduced serum medium as described under “Materials and Methods.” Expression of EMMPRIN was determined by Western blot (B), and fluorescence-activated cell sorter analysis (C). Fluorescence-activated cell sorter results were expressed as EMMPRIN expression in transfected cell lines as a percentage of expression in untransfected parental cells.
isothiocyanate-conjugated mouse anti-EMMPRIN antibody (1:10 dilution) and analyzed by flow cytometry.

**Hanging Drop Assay**—The hanging drop assay was performed as described previously (17), with minor modification. Briefly, cells were removed from confluent 10-cm plates with trypsin-EDTA, washed, counted, and resuspended at a concentration of $4 \times 10^6$ cells/ml in complete medium. Twelve-μl aliquots of this suspension were deposited on the underside of a 10-cm tissue culture dish lid. The lid was then inverted over 8 ml of phosphate-buffered saline to create hanging drops on the upper lid. Drops were incubated under tissue culture conditions for 2–3 days. Aggregate compaction was measured as follows. High contrast aggregate images were captured and digitized. Image analysis was performed using IP Lab imaging software (Slanalytics Inc., Fairfax, VA). Each image was adjusted for optimum contrast. Aggregates were then assigned as false color, and the outlines were automatically traced. The number of pixels underlying the outline was calculated by the imaging software.

**Assay of Fibronectin Matrix Assembly**—The assembly of high molecular weight fibronectin multimers was determined by a previously described method (17). Briefly, cell aggregates were lysed in DOC lysis buffer (2% sodium deoxycholate, 0.02 M Tris-HCl, pH 8.8, 2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 2 mM iodoacetic acid, and 2 mM N-ethylmaleimide), passed through a 26-gauge needle, and the DOC-insoluble components and DOC-insoluble components were separated. DOC-insoluble components were solubilized using SDS lysis buffer (1% SDS, 25 mM Tris-HCl, pH 8.0, 2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 2 mM iodoacetic acid, and 2 mM N-ethylmaleimide). Reduced lysates were resolved by SDS-PAGE, transferred to a 0.45-m nitrocellulose membrane, and probed with an anti-fibronectin antibody. Under reducing conditions, high molecular mass fibronectin multimers resolve at 220 kDa.

**Anoikis Assay**—Cells ($5 \times 10^5$ cells/2 ml/well) were plated onto standard 6-well tissue culture plates or poly-HEMA-coated plates (Corning Incorporated, Corning, NY) to prohibit attachment (18). After a 24- or 48-h incubation at 37 °C in a humidified atmosphere containing 5% CO$_2$, 95% air, the viability of cells was measured using trypan blue exclusion on a ViCell cell viability analyzer (Beckman Coulter, Fullerton, CA). Apoptosis was measured by the TUNEL assay and Western blot analysis of cleaved caspase-3. In the TUNEL assay, APO-BRDUTM kit (BioSource International, Inc., Camarillo, CA) was used to label and stain cells according to manufacturer’s protocol, and the samples were analyzed on a FC500 Beckman-Coulter flow cytometer. Active caspase-3, which is present in cells undergoing apoptosis, was detected by Western blot using polyclonal anti-caspase-3 antibody that recognizes the cleaved, active caspase-3.

**Western Blot Analysis**—Cell lysates were prepared in TNT buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1% aprotinin), and proteins were resolved
by SDS-PAGE. Transfer of proteins to nitrocellulose was performed by the method of Towbin \textit{et al.} (19). The blots were incubated in blocking solution consisting of 5% milk in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20 at room temperature for 1 h and then immunoblotted with the respective antibodies. Detection by enzyme-linked chemiluminescence (ECL) was performed according to the manufacturer’s protocol (Amersham Biosciences, Piscataway, NJ). \( \beta \)-Actin was used as a loading control.

**RESULTS**

\textbf{Generation and Characterization of EMMPRIN-targeted siRNA or shRNA Transfectants—} To assess the role of EMMPRIN in protecting cells from detachment-induced programmed cell death or anoikis, we utilized human breast cancer cell lines with different levels of EMMPRIN expression modulated by methods of overexpression, antisense, or RNA interference. EMMPRIN transfectants and antisense-transfected cells were described previously (6, 15). MDA-MB-231 cells treated with siRNA showed a 80–90% reduction in EMMPRIN expression 72 h after transfection, as compared with the cells transfected with a non-targeting RNA (Fig. 1A). We also constructed a vector that expressed an EMMPRIN-targeted shRNA and isolated stable transfect clones. As shown in Fig. 1B and C, in shRNA-expressing MDA-MB-231 cell line, EMMPRIN expression was decreased by greater than 80%, as determined by Western blot and flow cytometry.

\textbf{Effect of EMMPRIN on Cell Aggregate Formation—} We evaluated the effect of EMMPRIN on formation of cellular aggregates, the type of intercellular contacts known to affect sensitivity to anoikis (20, 21). In hanging drop cultures, we observed that breast carcinoma cells express-
ing different amounts of EMMPRIN formed multicellular aggregates with different degrees of compaction and size of surface areas (Fig. 2, A and B). Aggregates of cells with higher levels of EMMPRIN (parental or control vector-transfected MDA-MB-231 and MDA-MB-436 cells) had larger surface areas than those of the cells with lower levels of the protein (antisense- or shRNA-transfected cells) (Fig. 2B). Aggregates with larger surface areas contained more viable cells than those with smaller surface areas (Fig. 2C). These results imply a correlation between EMMPRIN expression, aggregate compaction, and cell survival. It was previously reported that cells cultured in “hanging drops” assembled a fibronectin matrix to support aggregate compaction (17). To gain insight into the mechanism of EMMPRIN-induced alterations of aggregate compaction, we assessed the effect of EMMPRIN expression on the assembly of fibronectin multimers by measuring insoluble fibronectin using a differential solubilization/Western blot analysis. As demonstrated in Fig. 3, high molecular weight, insoluble fibronectin increased in hanging drop aggregates of cells with silenced EMMPRIN expression. These cells also formed more compact aggregates as compared with the controls that expressed high level of EMMPRIN and formed less compact aggregates. These results indicate that EMMPRIN might affect aggregate compaction by regulating assembly of a fibronectin matrix.

Effect of EMMPRIN on Cell Viability in the Absence of Attachment—To ascertain whether EMMPRIN affects survival of carcinoma cells in

FIGURE 5. Expression of EMMPRIN inhibits anoikis. MDA-MB-231 cells transfected with EMMPRIN siRNA (A) or shRNA (B and D), and MDA-MB-436 cells transfected with an EMMPRIN expression vector (C) were seeded on standard 100-mm tissue culture dishes or poly-HEMA-coated dishes (1 × 10^6 cells/dish) and then incubated for 24-h at 37 °C in a humidified atmosphere containing 5% CO_2, 95% air. Apoptosis was assayed by measuring caspase-3 activation and DNA fragmentation. A–C, Western blot analysis of cleaved caspase-3. D, fluorescence-activated cell sorter plots of TUNEL assay. Results are representative of two similar experiments. FITC, fluorescein isothiocyanate.
EMMPRIN and Anoikis in Cancer

Expression of EMMPRIN Confers Resistance to Anoikis—To determine whether the survival-promoting function of EMMPRIN was through inhibition of anoikis, we compared the apoptotic properties of cell lines expressing different amounts of EMMPRIN. We found that the expression of EMMPRIN indeed decreased susceptibility of breast cancer cells to detachment-induced apoptosis as measured by caspase-3 activation and DNA fragmentation. As shown in Fig. 5, A and B, knockdown of EMMPRIN by either siRNA or shRNA resulted in activation of caspase-3 in MDA-MB-231 cells cultured in suspension on poly-HEMA but had no detectable effect on cells grown as monolayers attached to plastic. In contrast, when EMMPRIN was overexpressed by transfection, cleaved caspase-3 was barely detected when cells were cultured in suspension (Fig. 5C). The TUNEL assay also demonstrated that when cultured in suspension on poly-HEMA, there was a marked increase in DNA fragmentation in cells expressing EMMPRIN-targeted shRNA, as compared with parental or control vector-transfected cells (Fig. 5D). Similar to the results shown in Fig. 5, A and B, silencing of EMMPRIN expression did not cause apoptosis in the cells grown attached (Fig. 5D). These results indicate a specific role for EMMPRIN in inhibiting anoikis, the onset of apoptosis caused by loss of matrix attachment.

Down-regulation of Bim Mediates Resistance to Anoikis Conferred by EMMPRIN—We next explored the mechanism(s) involved in anoikis resistance provided by EMMPRIN. Toole and co-workers (13, 14) have shown that the MAP kinase (ERK1/2) pathway can be stimulated by EMMPRIN. Bim, a proapoptotic BH3-only protein that plays a crucial role in regulating anoikis (22), is phosphorylated by ERK1/2, and phosphorylation of Bim leads to its proteasomal degradation (23). Therefore, we asked whether resistance to anoikis in EMMPRIN-expressing breast carcinoma cells was associated with Bim down-regulation. We first compared the level of Bim in cells expressing different levels of EMMPRIN. As shown in Fig. 6A, Bim (Bim-EL, Bim-L, and Bim-S) levels were reduced, whereas phospho-ERK1/2 levels were increased, in EMMPRIN-transfected MDA-MB-436 cells grown as attached or suspended cultures, in comparison with the cells transfected with a control vector. In contrast, silencing of EMMPRIN expression led to an increase in accumulation of Bim and a decrease in phosphorylation of ERK1/2 (Fig. 6B). To directly assess the role of Bim in resistance to anoikis induced by EMMPRIN, we used siRNA to repress the expression of Bim in cells whose EMMPRIN was silenced (Fig. 7A). Knockdown of Bim decreased the ability of cells with depleted EMMPRIN expression to undergo detachment-induced apoptosis (Fig. 7B) and increased cell viability when cultured in suspension on poly-HEMA but did not affect cells grown attached to plastic (Fig. 7C).

MAP Kinase or Proteasome Inhibition Increases Bim Accumulation and Sensitivity to Anoikis—If EMMPRIN down-regulated Bim through the MAP kinase pathway, we reasoned that U0126, a selective MEK inhibitor, should block the effect. Fig. 8 shows that treatment of EMMPRIN transfectants grown in suspension with U0126 (50 μM) resulted in an increase in the accumulation of Bim proteins (Bim-EL, Bim-L, and Bim-S) in control cells and EMMPRIN transfectants. To corroborate that down-regulation of Bim in EMMPRIN-expressing cells was because of proteasomal degradation, we next treated cells with...
the proteasome inhibitor, epoxomicin. Fig. 8 shows that treatment of EMMPRIN transfectants grown in suspension with epoxomicin (100 nM) also increased Bim accumulation. Furthermore, the elevation of Bim by U0126 or epoxomicin was accompanied by an increased sensitivity to anoikis in control cells and EMMPRIN transfectants, as manifested by increased amounts of cleaved caspase-3 (Fig. 8).

**DISCUSSION**

EMMPRIN is a transmembrane glycoprotein that is abundant in various types of tumors and known to possess multiple functions, such as stimulating matrix metalloproteinase production (2), regulating calcium movement (4), inducing the expression of vascular endothelial cell growth factor (24), mediating cyclophilin activity (5), and modulating the expression of lactate transporters (3). The pathophysiological roles associated with those functions have also been reported. For example, overexpression of EMMPRIN was shown to promote tumorigenicity, invasion, and metastasis of malignant cells (6). Recently, EMMPRIN was found to enhance tumor angiogenesis both *in vitro* and *in vivo* (24). Here we report a mechanism by which EMMPRIN provides resistance of cancer cells to anoikis, a form of apoptosis induced by loss of matrix or cellular attachment and often defective in tumor cells that are of epithelial origin. We demonstrate in human breast cancer cells that expression of EMMPRIN diminishes anoikis (Figs. 5, 7, and 8), thereby increasing cell viability when anchorage is lost (Figs. 2 and 4). In addition, we show that the effect of EMMPRIN appears to result from MAP kinase-mediated down-regulation of a BH3-only proapoptotic protein, Bim (Figs. 6–8).

**FIGURE 7.** Bim-siRNA decreases anoikis and increases viability in MDA-MB-231 cells expressing EMMPRIN-shRNA. A, MDA-MB-231/EMMPRIN-shRNA or empty vector control cells were transfected with a Bim siRNA (100 nM) or scrambled RNA. Forty-eight h later, cell lysates were prepared from the transfected cells, and equal amounts (50 μg) of proteins were resolved by SDS-PAGE. Proteins were transferred to nitrocellulose, and Bim was detected by immunoblotting with a polyclonal anti-Bim antibody. β-Actin was used as a loading control. B, MDA-MB-231/EMMPRIN-shRNA or empty vector control cells were transfected with Bim siRNA (100 nM) or a scrambled RNA for 24 h, then plated onto standard 6-well tissue culture plates or poly-HEMA-coated plates and incubated for another 24 h. Apoptosis was measured by DNA fragmentation using TUNEL assay. FITC, fluorescein isothiocyanate. C, MDA-MB-231/EMMPRIN-shRNA or empty vector control cells transfected with Bim-siRNA were plated on poly-HEMA-coated plates and incubated for 24 h. Cell viability was measured by trypan blue exclusion. Results are representative of two similar experiments.
EMMPRIN and Anoikis in Cancer

Early evidence for a role of EMMPRIN in promoting tumor cell growth and invasion and metastasis was provided by Zucker et al. (6) through the use of EMMPRIN-transfected human breast cancer cells. These oncogenic and metastatic activities could be attributed to the recently demonstrated effects of EMMPRIN on hyaluronan and vascular endothelial growth factor production as well as its induction of matrix metalloproteinases (13, 14, 24). Here, we show that expression of EMMPRIN in cancer cells not only alters cell-cell interaction by modulating the assembly of fibronectin multimers (Figs. 2 and 3) but also blocks anoikis (Figs. 4 and 5). In the absence of anchorage, breast cancer cells expressing higher levels of EMMPRIN form multicellular aggregates with larger surface areas (Fig. 2), have higher viability (Fig. 4) and lower rates of apoptosis (Fig. 5), whereas knockdown of EMMPRIN expression produces the opposite effects (Figs. 2, 4, and 5). These data indicate that EMMPRIN may promote cancer cell survival by regulating intercellular contacts and inhibiting anoikis. The more pronounced effect of EMMPRIN knockdown on detachment-induced apoptosis measured by TUNEL assay of DNA fragments compared with caspase-3 activation likely reflects the fact that activation of caspase-3 is transient and that the cleaved product is turned over during apoptosis, whereas apoptotic cells with fragmented DNA persist for longer periods of time (25, 26). Tumor cells forming multicellular aggregates in suspension culture on poly-HEMA were reported to become more resistant to anoikis than single cells (20). Our data suggest that the surface area of hanging drop cellular aggregates is a phenotypic marker of cancer cells that are relatively resistant to anoikis, as cells that form larger and less compact aggregates are more resistant to anoikis than cells that form smaller and more compact aggregates. In addition, we demonstrate that larger, less compact aggregates in hanging drop cultures have decreased assembly of a fibronectin matrix than smaller, more compact aggregates (Fig. 3). These results are consistent with previous work that found that fibronectin matrix assembly correlates with aggregate compaction and cohesion (17). Decreased ERK phosphorylation has previously been shown to result in increased fibronectin matrix assembly (27). We also found that the silencing of EMMPRIN decreases ERK activity (Fig. 6B), consistent with the observed effects on fibronectin assembly. Recently, EMMPRIN has been reported to promote cytoskeletal rearrangements and affect cellular architecture in insect cells, and these effects are metalloproteinase-independent (28).

EMMPRIN can stimulate several survival pathways through action of hyaluronan. These pathways include AKT, focal adhesion kinase, and ERK (13, 14). In this study we identified Bim as a downstream effector of the EMMPRIN-MAP kinase pathway in suppressing anoikis. Bim, a BH3-only proapoptotic protein belonging to the Bcl-2 family, plays a critical role in triggering anoikis. For example, inhibition of Bim induction has been found to be the mechanism of anoikis deficiency caused by G1/S cell cycle arrest (29). In addition, Bim has also been shown to be a tumor suppressor and a key determinant of paclitaxel sensitivity in epithelial tumors (30). Our results demonstrate a converse relationship between EMMPRIN expression and Bim accumulation, i.e. Bim is reduced in cells with high expression of EMMPRIN and suppression of EMMPRIN up-regulates Bim (Fig. 6). Our results also provide more direct evidence for a role for Bim in EMMPRIN-induced resistance to anoikis, as knockdown of Bim expression decreases detachment-triggered apoptosis and increases viability in cells whose expression of EMMPRIN is silenced by shRNA (Fig. 7). Bim is known to be phosphorylated by MAP kinase (ERK1/2), and phosphorylation of Bim results in its proteasomal degradation (23). Indeed, our results show that the activity of ERK1/2 in EMMPRIN-overexpressing cells is increased (Fig. 5), suggesting that the inhibition of Bim accumulation by EMMPRIN is because of phosphorylation of Bim by the MAP kinase pathway and degradation of the protein by the proteasome. This hypothesis is further supported by the use of a MEK inhibitor (U0126) and a proteasome inhibitor (epoxomicin), both of which can elevate Bim levels and enhance anoikis in EMMPRIN-expressing cells (Fig. 8). The decreases in phospho-ERK1/2 in cells treated with epoxomicin (Fig. 8) is likely because of the induction of MAP kinase phosphatases by the proteasome inhibitor, as previously observed by others (31, 32). Taken together, these results indicate that the down-regulation of Bim may account for anoikis resistance seen in EMMPRIN-expressing cells. How EMMPRIN stimulates the MAP kinase pathway remains an area of active investigation.

The acquisition of resistance to anoikis is thought to be a crucial step in neoplastic or metastatic transformation (33, 34). Nevertheless, the molecular mechanisms involved in anoikis resistance are not well understood. Recently, alternations of death receptors, Src, laminins, and phosphoinositide-3 kinase signaling have been implicated (33, 35–37). The present study, which identified EMMPRIN as a suppressor of anoikis, reveals a new pathway to regulate anoikis (Fig. 9). Whether the EMMPRIN-initiated pathway is impinged upon by other signaling pathways requires further investigation. Because the ability of tumor cells to invade/metastasize and to develop resistance to treatment represents two critically important malignant characteristics, and defective anoikis likely contributes to both of these features, re-establishing anoikis sensitivity may be a novel therapeutic strategy for cancers. The findings by us and others (14) that EMMPRIN acts as an anoikis suppressor provides further evidence that this protein may be a novel target for cancer therapy.
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