Published OnlineFirst February 1, 2008; DOI: 10.1158/1541-7786.MCR-07-0254

Mesothelin Promotes Anchorage-Independent Growth and Prevents Anoikis via Extracellular Signal-Regulated Kinase Signaling Pathway in Human Breast Cancer Cells

Norihisa Uehara, Yoichiro Matsuoka, and Airo Tsubura

Second Department of Pathology, Kansai Medical University, Moriguchi, Japan

Abstract

Mesothelin (MSLN) is a glycoprotein that is overexpressed in various tumors. MSLN is present on the cell surface and is also released into body fluids or culture supernatants from MSLN-positive tumor cells. Despite intensive study of MSLN as a diagnostic marker or target for immunotherapy, its biological function is largely unknown. In the present study, we examined the effects of ectopic expression of MSLN in human breast cancer cell lines (MCF-7, T47D, and MDA-MB-231). We found that overexpression of MSLN promoted anchorage-independent growth in soft agar. In addition, MDA-MB-231 cells expressing high levels of MSLN exhibited resistance to anoikis (a type of apoptosis induced by detachment from substratum), as indicated by decreased DNA fragmentation and down-regulation of the proapoptotic protein Bim. Incubating MSLN-expressing MDA-MB-231 cells in the presence of U0126, an inhibitor of mitogen-activated protein/extracellular-signal-regulated kinase kinase, induced accumulation of Bim and restored susceptibility to anoikis. Western blot analysis also revealed that overexpression of MSLN resulted in sustained activation of extracellular signal-regulated kinase 1/2 and suppression of Bim. The present results constitute novel evidence that MSLN enables cells to survive under anchorage-independent conditions by suppressing Bim induction via the extracellular signal-regulated kinase signaling pathway. (Mol Cancer Res 2008;6(2):186–93)

Introduction

Mesothelin (MSLN) is a 40-kDa glycosylphosphatidylinositol-linked cell surface antigen that is present on normal mesothelial cells, and that is overexpressed in mesotheliomas, pancreatic cancers, ovarian cancers, and some other cancers (1). The mesothelin gene (*MSLN*) encodes a 69-kDa precursor protein that is processed to a 40-kDa membrane-bound protein (MSLN) and a ~30-kDa soluble protein. The soluble product, which is known as megakaryocyte-potentiating factor, stimulates megakaryocyte colony-forming activity (2, 3). Recent studies indicate that in patients with MSLN-positive ovarian carcinoma, membrane-bound MSLN is released from the carcinoma into body fluids (4). Detection of soluble MSLN-related protein is a promising method for diagnosing tumors that express MSLN. Membrane-bound MSLN is an attractive target for immunotherapy due to the limited distribution of MSLN in normal tissues and the elevated expression of MSLN in several human tumors. SS1P is an anti-MSLN immunotoxin that was developed by Chowdhury's group (5); phase I clinical studies of SS1P are currently being conducted. Recent studies indicate that the tumor marker CA125/MUC16 binds to MSLN at the cell surface of ovarian carcinoma cell line OVCAR-3, suggesting that interaction between CA125/MUC16 and MSLN plays a role in heterotypic cell adhesion and metastatic cell spreading (6, 7). In spite of this interest, the biological significance of MSLN expression is not well understood. Indeed, MSLN-deficient mice exhibit no anatomic or reproductive abnormalities (8).

Overexpression of MSLN has been observed in several human tumors, including breast carcinoma (9, 10). Further, clues to the role of MSLN come from epidemiologic studies indicating that full-term pregnancy at a young age results in significant reduction of the lifetime risk of breast cancer compared with nulliparous women (11, 12). Parity-induced protection against mammary cancer has also been observed in rats (13, 14). In a previous study using oligonucleotide microarray analysis, we found that the *MSLN* gene was significantly down-regulated in parous mammary glands of rats compared with age-matched virgins (15). Several studies based on animal or cell culture models indicate that MSLN expression is involved in the Wnt or β-catenin signaling pathway, whose deregulation plays an important role in carcinogenesis (16-18).

In light of evidence suggesting that MSLN overexpression contributes to mammary carcinogenesis, we generated MSLN-expressing human breast cancer cell lines (MCF-7, T47D, and MDA-MB-231) to examine the effects of MSLN expression on two cancer phenotypes, anchorage-independent growth and survival. Three breast cancer cell lines were used—MCF-7, T47D, and MDA-MB-231. These were selected for this study because of their different characteristics of cell phenotype (19).

Received 6/5/07; revised 9/21/07; accepted 10/1/07.
Grant support: Grant-in-Aid for Young Scientists (B) 18790947 (N. Uehara) and a Fujita Memorial Fund for Medical Research from the Japan Society for the Promotion of Science (N. Uehara).
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Requests for reprints: Norihisa Uehara, Second Department of Pathology, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi-shi, Osaka 570-8506, Japan. Phone: 81-6-6993-9432; Fax: 81-6-6992-5023. E-mail: ueharan@takii.kmu.ac.jp

doi:10.1158/1541-7786.MCR-07-0254

Published OnlineFirst February 1, 2008; DOI: 10.1158/1541-7786.MCR-07-0254

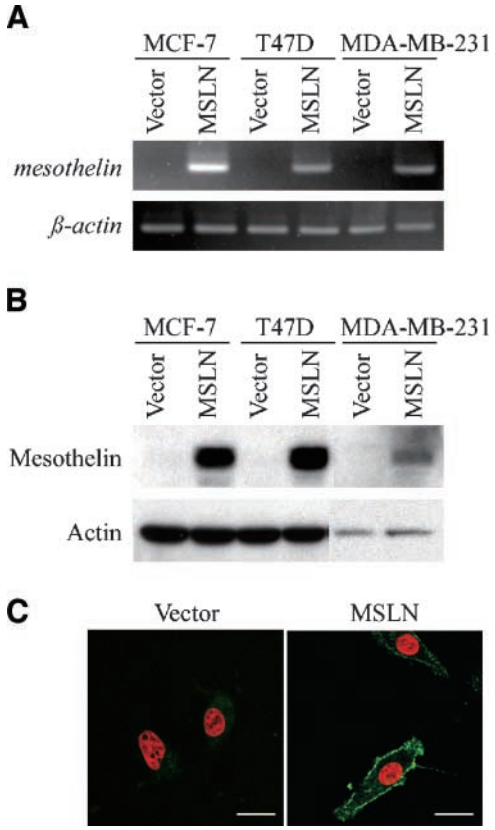

FIGURE 1. Stable expression of MSLN in breast cancer cells. **A.** Reverse transcription-PCR analysis of total RNA (1 μg) isolated from vector control and *MSLN*-transfected breast cancer cells. *β-Actin* was used as a loading control. **B.** Whole-cell lysates from vector control and *MSLN*-transfected breast cancer cells were subjected to SDS-PAGE and immunoblotted with anti-MSLN antibody. Actin was used as a loading control. **C.** Detection of exogenous MSLN by confocal laser microscopy. Immunofluorescence images of vector control (*left*) and MSLN-transfected (*right*) MDA-MB-231 cells. Note that in *MSLN*-transfected cells, MSLN localizes primarily at the plasma membrane (*green*). Cells were counter-stained with 4′,6-diamidino-2-phenylindole (*red*). Bar, 20 μm.

MCF-7 (20) and T47D (21), steroid receptor–positive cell lines, retain a luminal phenotype originating from the epithelial lumen, whereas MDA-MB-231, a steroid receptor–negative cell line, has a highly invasive capacity and display some basal/myoepithelial characteristics typical of those originating from the basal layer of the epithelium (22).

In the present study, we report that overexpression of MSLN in breast cancer cells facilitated anchorage-independent growth and conferred resistance to anoikis, a form of apoptosis induced by detachment from the substratum. Furthermore, the resistance to anoikis conferred by MSLN overexpression correlated with suppression of the proapoptotic protein Bim via the extracellular signal-regulated kinase (ERK) signaling pathway.

Results

Generation of MSLN-Expressing Breast Cancer Cells

MCF-7, T47D, and MDA-MB-231 human breast cancer cells were transfected with a pcDNA mammalian expression vector containing full-length cDNA encoding human *MSLN,* or with the empty pcDNA vector. After 4 weeks of selection with G418, MSLN-expressing cells and vector control cells were obtained for each of the three breast cancer cell lines. MSLN mRNA and protein expression were measured by reverse transcription-PCR and Western blot analysis (Fig. 1A and B). All three MSLN-expressing cell lines (MCF-7/MSLN, T47D/MSLN, and MDAMB231/MSLN) expressed high levels of exogenous MSLN mRNA and protein (~40 kDa), whereas none of the three vector control cell lines (MCF-7/vector, T47D/vector, and MDAMB231/vector) expressed detectable levels of exogenous MSLN mRNA or protein (Fig. 1A and B). MSLN localization in MDAMB231/MSLN and MDAMB231/vector cells was monitored by immunocytochemistry. In MDAMB231/MSLN, exogenous MSLN localized at the plasma membrane, whereas no signal was detected in MDAMB231/vector cells (Fig. 1C).

MSLN Expression Promotes Anchorage-Independent Growth but not Anchorage-Dependent Growth

To evaluate the effects of MSLN overexpression on cell proliferation, we used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on the vector control cell lines and the MSLN-expressing cell lines in standard monolayer cultures (Fig. 2). There was no significant difference in exponential monolayer growth between any of the MSLN-expressing cell lines and its respective vector control cell line (Fig. 2). To ascertain whether MSLN overexpression affected anchorage-independent growth, we assessed the ability of MSLN-expressing cells to form colonies in soft agar (Fig. 3). After 15 days of culture, image analysis revealed that each of the MSLN-expressing cell lines had formed a significantly greater number (~1.5-fold) of colonies that were of a larger size (~1.9-fold) when compared with its respective vector control cell line (Fig. 3).

Effect of MSLN Expression on Cell Viability under Anchorage-Independent Conditions

Because MSLN expression promoted colony formation in soft agar (Fig. 3), we next examined whether MSLN expression promoted survival in the absence of matrix attachment. Cell viability and growth rate were assessed for 72 h of culture either as monolayers attached to plastic or in suspension in Corning Ultra-Low-Attachment plates. For cells grown as attached monolayers, there was no significant difference in viability and growth rate between the MSLN-expressing cell lines and their

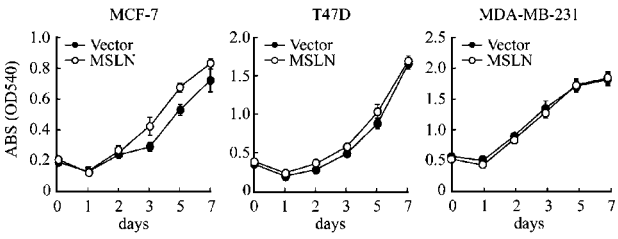

FIGURE 2. MSLN does not affect anchorage-dependent growth of breast cancer cells. Vector control (●) and *MSLN*-transfected (○) breast cancer cells were plated on 96-well plates at 5×10^3 per well. The cells were cultured for the indicated number of days, and viable cells were identified by MTT assay, as described in Materials and Methods. Points, mean of three independent experiments; bars, SD.

Published OnlineFirst February 1, 2008; DOI: 10.1158/1541-7786.MCR-07-0254

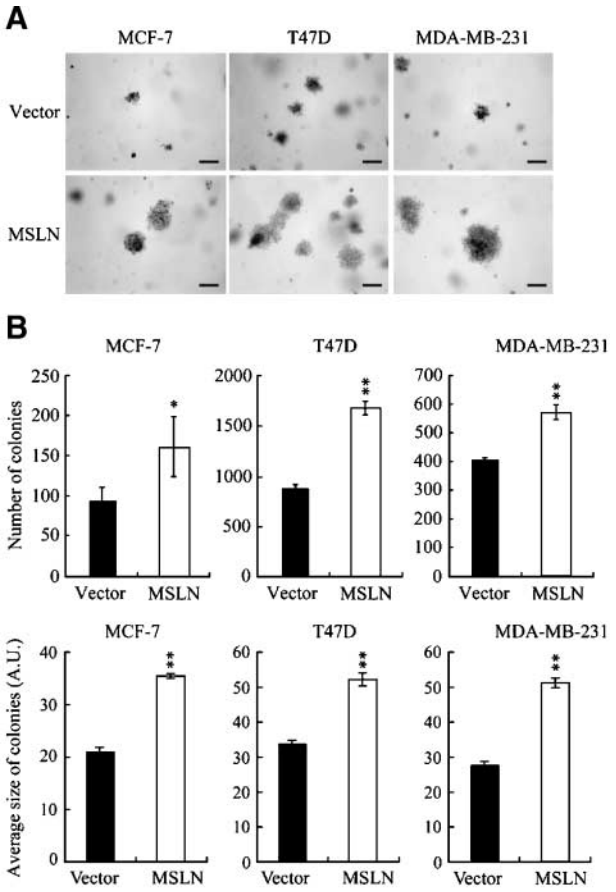

FIGURE 3. MSLN promotes anchorage-independent growth. **A.** Photomicrographs show representative colony formation from vector control and *MSLN*-transfected breast cancer cells grown in soft agar. Bar, 200 μm. **B.** Numbers and sizes of colonies formed by vector control and MSLN-expressing breast cancer cell lines. Columns, mean (three wells per transfectant from two separate experiments); bars, SD. *, $P < 0.05$. **, $P < 0.01$.

respective vector control cell lines (data not shown). In contrast, after 72 h of culture in suspension, each of the MSLN-expressing cell lines exhibited significantly greater viability than its respective vector control cell line (Fig. 4A). In addition, each of the vector control cell lines exhibited either no change or only a slight increase in total cell number, whereas all three MSLN cell lines cultured in suspension continued to proliferate. In particular, the total number of MDAMB231/MSLN cells was significantly greater than that of MDAMB231/vector cells (Fig. 4A and B). These results indicate that MSLN expression promotes not only cell survival but also cell proliferation under anchorage-independent conditions.

MSLN Expression Prevents Anoikis and Suppresses Bim Accumulation under Anchorage-Independent Conditions

To examine whether the survival-promoting effect of MSLN under anchorage-independent conditions was due to prevention of anoikis, we did DNA fragmentation analysis of MDAMB231/MSLN and MDAMB231/vector cell lines. Abnormally high levels of DNA fragmentation have been observed in cells undergoing anoikis (23). After 72 h of culture in suspension, MDAMB231/vector cells exhibited a higher level of DNA fragmentation than MDAMB231/MSLN cells (Fig. 5A).

Cells that express high levels of antiapoptotic proteins such as Bcl-2 and Bcl-X_L are highly resistant to anoikis (24). In contrast, it has been reported that the proapoptotic protein Bim plays an important role in induction of anoikis (25-27). To investigate the mechanisms by which MSLN expression may protect breast cancer cells from anoikis, we examined Bim and Bcl-2 levels. Representative Western blots for Bim and Bcl-2 are shown in Fig. 5B, and the quantitation of Bim levels under various conditions is summarized in Fig. 5C. Upon detachment from the substratum, MDAMB231/vector cells exhibited marked accumulation of Bim protein, whereas no such accumulation of Bim was observed in MDAMB231/MSLN cells (Fig. 5B). Moreover, MSLN-expressing MDA-MB-231 cells cultured in suspension exhibited a mobility shift in Bim that is considered indicative of phosphorylation, whereas no such mobility shift in Bim occurred in the non–MSLN-expressing control MDA-MB-231 cells (Fig. 5B). In contrast, there was no difference in Bcl-2 levels between MDAMB231/MSLN and MDAMB231/vector cells (Fig. 5B).

MSLN-Induced Resistance to Anoikis Is Mediated by the ERK1/2 Signaling Pathway

Studies indicate that ERK can promote cell survival by regulating Bim function via phosphorylation followed by proteasome degradation (28, 29). To investigate whether MSLN-induced resistance to anoikis is mediated by the ERK

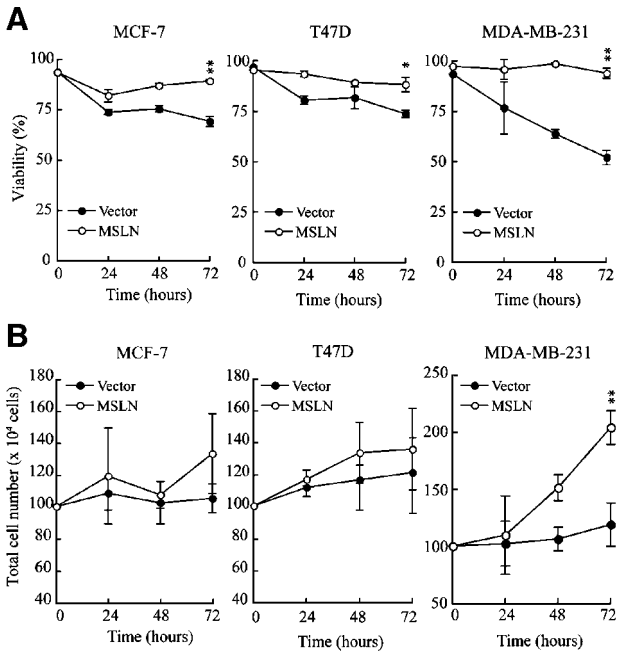

FIGURE 4. MSLN promotes cell survival and proliferation under anchorage-independent conditions. Vector control and MSLN-expressing breast cancer cells were plated on six-well Coaster Ultra Low Attachment plates at 1×10^6 per well. At each time point, viability (**A**) and total cell number (**B**) were counted by trypan blue exclusion. Points, mean; bars, SD. *, $P < 0.05$. **, $P < 0.01$.

Published OnlineFirst February 1, 2008; DOI: 10.1158/1541-7786.MCR-07-0254

signaling pathway, we examined the effects of the selective mitogen-activated protein/extracellular-signal-regulated kinase kinase (MEK) inhibitor U0126 on anoikis in MDAMB231/MSLN and MDAMB231/vector cells (Fig. 6A). MDAMB231/MSLN cells exhibited greater resistance to anoikis (2.0-fold) and exhibited an increase in total cell number (2.0-fold) compared with MDAMB231/vector cells. U0126 reduced cell viability and cell number significantly and to the same extent in both MDAMB231/MSLN and MDAMB231/vector cells.

We next examined whether MSLN-induced resistance to anoikis correlated with activation of ERK and reduction in Bim level. Representative Western blots for phosphorylated ERK and Bim are shown in Fig. 6B, and quantitative data are summarized in Fig. 6C. After detachment from the substratum, MDAMB231/vector cells exhibited a decreased level of phosphorylated ERK1/2, whereas MDAMB231/MSLN cells maintained a higher level of phosphorylated ERK1/2, which inversely correlated with Bim levels. In both MDAMB231/MSLN and MDAMB231/vector cells, U0126 reduced both the level of phosphorylated ERK1/2 and the accumulation of Bim (Bim-$_{EL}$, Bim-$_{L}$ and Bim-$_{S}$) that are typically observed after detachment. Under anchorage-independent conditions, MSLN overexpression is correlated with sustained activation of ERK1/2 and suppression of Bim, which correlated with a high level of cell viability and proliferation (Fig. 6). U0126 induces a marked decrease in total ERK1/2 levels in MDAMB231/MSLN cells than in MDAMB231/vector cells. Moreover, U0126 induces a marked increase in Bim-$_{EL}$ levels in both MSLN-expressing and vector control cells (Fig. 6B and C). These results indicate that decrease in Bim levels may not be a major event resulting from MSLN overexpression per se.

Discussion

The present results indicate that MSLN may have a novel function as a mediator of cell survival under anchorage-independent conditions. They show that MSLN is localized on the cell surface and that its overexpression in breast cancer cells is implicated in promoting anchorage-independent growth and preventing anoikis. They also show that MSLN-induced resistance to anoikis is associated with sustained activation of ERK1/2 and suppression of the levels of proapoptotic protein Bim in MDA-MB-231 cells.

Normal adherent cells are strongly dependent on adhesion to extracellular matrix for cell proliferation and undergo apoptosis if they are detached from the substratum, in a process known as anoikis (30). In contrast, tumor cells can survive and grow without adhesion to a substratum; this is a critical factor in tumorigenic transformation (30, 31). Thus, acquisition of resistance to anoikis has important implications for cancer metastasis (32). In the present study, all three MSLN-expressing breast cancer cell lines (MCF-7/MSLN, T47D/MSLN, and MDAMB231/MSLN) exhibited increased anchorage-independent growth when cultured in soft agar (Fig. 3) and increased cell viability and proliferation when cultured in suspension (Figs. 4 and 6A), compared with their respective vector control cell lines. Although the increased growth rate of MCF-7/MSLN and T47D/MSLN cells cultured in suspension was not statistically significant, the increase exhibited by MDAMB231/MSLN cells was (Fig. 4A). This may be due to differences in the specific cell line context of effectors of the MSLN signaling pathway. In a previous study, these three cell lines were found to have different levels of expression of ERK, AKT, and Ras, which are implicated in cell survival and proliferation (33). Because MDAMB231/MSLN cells exhibited marked resistance to anoikis in the present study (Fig. 4), and because many studies of anoikis have been conducted in MDA-MB-231 cells (23, 25, 27, 33-35), we used MDA-MB-231 cells for

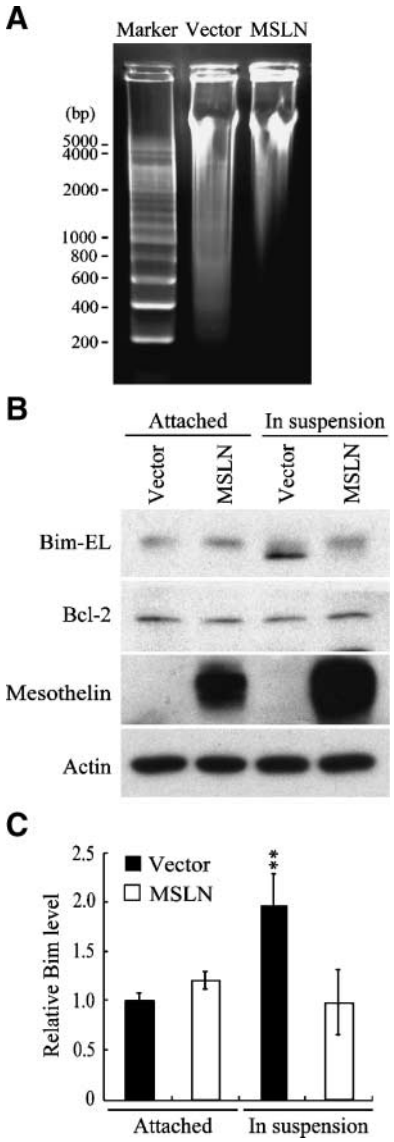

FIGURE 5. MSLN inhibits anoikis and suppresses Bim levels under anchorage-independent conditions. **A.** DNA fragmentation assay of vector control and MSLN-expressing MDA-MB-231 cells under anchorage-independent conditions. **B.** Representative data for Bim and Bcl-2 levels. Vector control and MSLN-expressing MDA-MB-231 cell lysates were prepared from monolayer or cultures in suspension (3 d after detachment) and were subjected to Western blot analysis. **C.** Protein level was normalized to actin, and the Bim level is shown relative to the value for vector control cells cultured under attached conditions (normalized at 1). Columns, mean; bars, SD. **, $P < 0.01$ compared with vector control cells cultured under attached conditions.

Published OnlineFirst February 1, 2008; DOI: 10.1158/1541-7786.MCR-07-0254

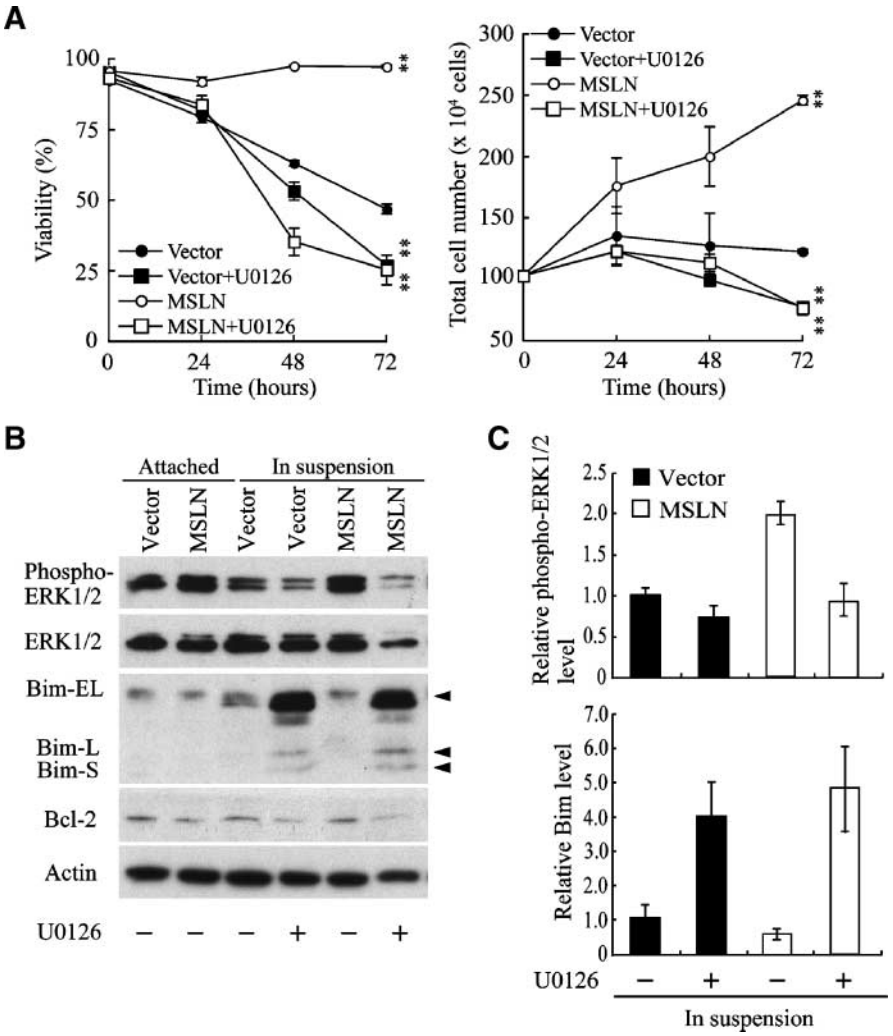

FIGURE 6. Effect of MEK-1 inhibitor U0126 on cell viability and proliferation, and levels of phosphorylated ERK1/2, Bim, and Bcl-2 in MSLN-expressing MDA-MB-231 cells. **A.** Vector control and MSLN-expressing MDA-MB-231 cells were plated on six-well Ultra Low Attachment plates at 1×10^6 per well with or without U0126 (10 μmol/L). At each time point, viability and total cell number were counted by trypan blue exclusion. **, $P < 0.01$ compared with untreated vector control cells. **B.** Representative data for phosphorylated-ERK1/2, Bim, and Bcl-2 levels. Vector control and MSLN-expressing MDA-MB-231 cell lysates were prepared from cultures in suspension (3 d after detachment) grown with or without U0126, and were subjected to Western blot analyses. **C.** For phosphorylated ERK1/2 and Bim, the total protein level in the sample was normalized to ERK1/2 and actin, respectively, and the protein level is shown relative to the value for respective vector control cells (normalized at 1). Columns, mean; bars, SD.

further investigating the mechanisms by which MSLN prevents anoikis.

The proteins of the Bcl-2 family play a central role in regulating apoptosis (36). Bim, the proapoptotic protein consisting of a single BH3 domain, has been shown to play a critical role in induction of anoikis (25-27, 37). The present results indicate that the resistance to anoikis observed in MSLN-expressing MDA-MB-231 cells correlates with reduction in Bim levels (Fig. 6). Bim can promote apoptosis by heterodimerizing and neutralizing antiapoptotic proteins such as Bcl-2 and Bcl-X_L, resulting in activation of Bax (38). Overexpression of Bcl-2 is associated with strong resistance to anoikis (24), but the present results indicate that the Bcl-2 level is not affected by MSLN expression under anchorage-independent conditions (Figs. 5B and 6B). Recent studies indicate that phosphatidylinositol 3-kinase, FAK, and ERK signaling play important roles in the regulation of anoikis (39). In particular, several recent studies clearly show that in MCF-10A immortalized breast epithelial cells and MDA-MB-231 breast cancer cells, activation of ERK inhibits anoikis via regulation of Bim (25-27). Furthermore, phosphorylation of Bim by ERK1/2 promotes proteosomal degradation, leading to attenuation of the proapoptotic activity of Bim (28, 29). The present results show that MSLN-overexpressing cells exhibit sustained phosphorylation of ERK1/2, whereas the non–MSLN-expressing control cells exhibit a lower level of ERK1/2 phosphorylation upon detachment from substratum; this decreased phosphorylation in control cells coincides with an accumulation of Bim (Fig. 6B and C). They also show that activation of ERK1/2 in MSLN-expressing MDA-MB-231 cells cultured in suspension results in a mobility shift in Bim indicative of phosphorylation, whereas no such mobility shift in Bim occurred for non–MSLN-expressing control MDA-MB-231 cells (Figs. 5B and 6B). These results indicate that suppression of Bim levels in MSLN-expressing MDA-MB-231 cells may be due to proteosomal degradation following phosphorylation of Bim by ERK1/2. Furthermore, the MSLN-induced resistance to anoikis identified here and the concomitant reduction in Bim levels are dependent on ERK activity, because the specific MEK inhibitor U0126 decreases the level of phosphorylated ERK1/2, increases Bim level, and decreases cell viability (Fig. 6). It has also been reported that the activation of ERK stimulates cell proliferation (40). MSLN overexpression results in the sustained phosphorylation of

Published OnlineFirst February 1, 2008; DOI: 10.1158/1541-7786.MCR-07-0254

ERK1/2 and suppresses the increase in Bim level associated with detachment. This enables cells not only to survive but also to proliferate under anchorage-independent conditions (Figs. 4 and 6A). Thus, MSLN overexpression may contribute to cell survival and proliferation that may result in the malignant and metastatic phenotype in MSLN-positive tumor cells. U0126 treatment induces anoikis sensitivity and a significant increase in the level of Bim protein in MDA-MB-231 cells under anchorage-independent conditions (25). Similarly, U0126 treatment results in a significant accumulation of Bim in both MSLN-expressing and vector control cells (Fig. 6B and C). In addition, U0126 induced a significant decrease in the total ERK1/2 level in MDAMB231/MSLN cells compared with MDAMB231/vector cells (Fig. 6B). Although U0126 is the selective inhibitor of MEK, off-target effects cannot be ruled out. Use of MEK1/2 gene knockdown might further elucidate mechanisms by which MSLN promotes cell survival and proliferation via the ERK signaling pathway.

Studies show that phosphorylation of phosphatidylinositol 3-kinase and FAK also plays a crucial role in resistance to anoikis (41, 42). However, in preliminary experiments related to the present study, the specific phosphatidylinositol 3-kinase inhibitor LY294002 did not affect the viability of MDAMB231/MSLN cells; this finding is consistent with the results of the accompanying preliminary Western blot analysis, which showed that MSLN did not affect the phosphorylation of either phosphatidylinositol 3-kinase or FAK (data not shown).

Recent studies of patients with ovarian carcinoma indicate that MSLN is released from the carcinoma into ascites (4). This phenomenon has also been observed *in vitro* using an MSLN-transfected A431 human epidermoid carcinoma cell line (43). In accordance with these previous reports, in the present study MSLN was localized at the cell surface of MDAMB231/MSLN cells (Fig. 1), and those cells released MSLN into the cell culture supernatant (data not shown). However, the MDAMB231/MSLN cell culture supernatant, which contained the soluble form of MSLN, did not affect either anoikis or ERK activation (data not shown). Further study is needed to elucidate the various functions of the soluble form of MSLN under different conditions.

In conclusion, the present findings provide evidence of a novel biological function for MSLN and a mechanism by which MSLN prevents anoikis in human breast cancer cells by down-regulating Bim via activation of the ERK signaling pathway. It would be interesting to determine whether other MSLN-positive cancer cells also exhibit resistance to anoikis via stimulation of the ERK pathway. The overexpression of MSLN observed in various tumors may be relevant to their malignant and metastatic phenotype. Further investigation is needed to determine how MSLN activates the ERK signaling pathway. Such findings could provide new insights into cancer therapy.

Materials and Methods

Cell Lines and Materials

The human breast cancer cell lines MCF-7, T47D, and MDA-MB-231 were maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum (Sigma) at 37°C in a 95% air/5% CO_2 atmosphere. The MEK1/2 inhibitor U0126 was obtained from Cell Signaling Technology. A stock solution of U0126 (10 mmol/L) was prepared using DMSO as the solvent.

Construction of MSLN Expression Plasmids and Transfection

The full-length cDNA for human *MSLN* variant 1 (*MSLN*, Genbank accession no. NM 005823) was amplified by PCR from HeLa cDNA, using the following primers: *MSLN*-forward, 5′-GCCAATCACCCTGCACATCAGAGTT-3′; *MSLN*-reverse, 5′-TTCCCGTTTACTGAGCGCGAGTTCT-3′. The amplified *MSLN* fragment was ligated into the pCRII vector (Invitrogen), released after digestion with the restriction enzymes *Hin*dIII and *Not*I, and ligated into the pcDNA3.1 mammalian expression vector (Invitrogen). The sequence of the cloned *MSLN* plasmids was verified by DNA sequencing. The *MSLN* expression plasmids were transfected into breast cancer cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. MCF-7, T47D, and MDA-MB-231 cells stably expressing *MSLN* were selected in the presence of 800 μg/mL G418 (Sigma).

Reverse Transcription-PCR Analyses

Total RNA from MCF-7, T47D, and MDA-MB-231 cells were prepared using the RNeasy Mini kit (Qiagen). cDNAs were synthesized from 1 μg of total RNA using the Superscript III first-strand synthesis kit (Invitrogen). *MSLN* mRNA expression in *MSLN*-transfected breast cancer cells was verified by performing reverse transcription-PCR using the following primers: 5′-GCCAATCACCCTGCACATCAGAGTT-3′ (forward); 5′-TTCCCGTTTACTGAGCGCGAGTTCT-3′ (reverse). *β-Actin* was also amplified with primers 5′-AGAAAATCTGGCACCACACC-3′ (forward); 5′-AGAGGCGTACAGGGATAGCA-3′ (reverse), and used as an internal control for equal loading. PCR amplification was done using the Platinum PCR supermix (Invitrogen), and the PCR conditions were as follows: after denaturing at 94°C for 1 min, 27 cycles of 15 s at 94°C, 15 s at 60°C, and 30 s at 72°C.

Western Blotting

Cell lysates were prepared by homogenization in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1% NP40 and 0.1% SDS, supplemented with 1 mmol/L phenylmethylsulfonyl fluoride]. The homogenates were incubated on ice for 30 min, and were then centrifuged at 15,000 × *g* for 15 min at 4°C, and the pellets were discarded. The protein concentration of the supernatants was measured using the detergent-compatible protein assay kit (Bio-Rad). Aliquots of lysates equivalent to 20 μg of protein were subjected to SDS-PAGE, followed by transfer to Hybond-P polyvinylidene difluoride membranes (Amersham Biosciences). The membranes were blocked with 5% nonfat powdered milk before incubation with the primary antibody. Then, the blots were washed and incubated with the appropriate secondary antibody coupled to horseradish peroxidase. The antigen-antibody complexes were detected using the enhanced chemiluminescence plus reagent (Amersham Biosciences). The following primary antibodies were used: anti-MSLN antibody (clone 5B2, Novocastra),

Published OnlineFirst February 1, 2008; DOI: 10.1158/1541-7786.MCR-07-0254

anti-Bim antibody (Sigma), anti–Bcl-2 antibody (Cell Signaling Technology), anti–phospho-p44/42 mitogen-activated protein kinase antibody (Thr^{202}/Tyr^{204}; Cell Signaling Technology), anti-ERK1 antibody (C-16, Santa Cruz Biotechnology), and horseradish peroxidase–conjugated anti-actin antiserum (Santa Cruz Biotechnology).

Cell Proliferation Assay

Cells were seeded at 5×10^3 per well on 96-well plates in growth medium supplemented with 10% serum, and were cultured in a humidified chamber at 37°C for up to 7 days. Viable cells were identified using the MTT assay. Briefly, the MTT reagent (5 mg/mL PBS) was added to each well, followed by incubation for 3 h at 37°C. The supernatants were aspirated, and the reaction was terminated by adding DMSO. The contents of the plates were mixed for 5 min, and the absorbance was read at 540 nm using a plate reader.

Soft Agar Colony Formation Assay

Anchorage-independent growth was assessed by monitoring colony formation in soft agar. First, 0.5% agarose in growth medium was added to a six-well plate and allowed to solidify. Then, 1×10^4 cells per well were plated in triplicate in 0.3% agarose onto the bottom agarose. The cells were incubated at 37°C in a 5% CO_2 atmosphere for 15 days. Fresh growth medium (0.5 mL/well) was added after 1 week of incubation. At the end of incubation, colonies were stained with 0.005% crystal violet for 1 h and photographed. Colonies were counted using the Image J imaging software developed at NIH.

Anoikis Assay

Cells were grown to subconfluence in 75-cm^2 tissue culture flasks. For cultures in suspension, cells were detached from the tissue culture flasks using 0.25% trypsin-EDTA (Invitrogen). Once detached, cells were cultured in complete medium on a six-well plate (1×10^6 per well) coated with Ultra Low Attachment Surface (Corning). The growth rate and viability of cells cultured in suspension were measured using the trypan blue exclusion assay. For the MEK inhibition experiment, U0126 was added to the culture medium to a final concentration of 10 μmol/L.

DNA Fragmentation Assay

After culturing the cells for 72 h in suspension, DNA was isolated by the digestion of cells with proteinase K at a concentration of 0.2 μg/μL in cell lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA (pH 8.0), 0.5% SDS] at 50°C for 2 h. RNase A was then added to the lysate to a final concentration of 0.2 μg/μL and incubated for 1 h. This was followed by extraction with phenol/chloroform. The DNA concentration was determined using DU640 spectrophotometer (Beckman Coulter). Ten micrograms of DNA were analyzed by electrophoresis in 1.5% (w/v) agarose gel and visualized under UV light after staining with ethidium bromide.

Immunofluorescence Analysis

MDA-MB-231 cells grown on a 35-mm glass-bottomed dish (Matsunami Glass) were washed with PBS, followed by fixation in 10% buffered formalin for 30 min at room temperature. The cells were then washed with TBS and blocked in TBS containing 5% bovine serum albumin at room temperature for 30 min. The cells were then incubated for 1 h with the anti-MSLN primary antibody (5B2, Novocastra), followed by repeated washes. The cells were then incubated for 1 h with the secondary antibody (Alexa Fluor 488 anti-mouse IgG; Molecular Probes). The cells were then counterstained with 4′,6-diamidino-2-phenylindole (Dojindo Laboratories) for 5 min. Cells were imaged using a laser scanning microscope (LSM510-META, Zeiss).

Statistical Analysis

All discrete values, expressed as mean ± SD, were analyzed using Student's t test. P values <0.05 and <0.01 were considered as significant and highly significant, respectively.

Acknowledgments

We thank Ms. T. Akamatsu for her technical assistance.

References

1. Chang K, Pastan I. Molecular cloning of mesothelin, a differentiation antigen present on mesothelium, mesotheliomas, and ovarian cancers. Proc Natl Acad Sci U S A 1996;93:136–40.

2. Kojima T, Oh-eda M, Hattori K, et al. Molecular cloning and expression of megakaryocyte potentiating factor cDNA. J Biol Chem 1995;270:21984–90.

3. Yamaguchi N, Yamamura Y, Konishi E, et al. Characterization, molecular cloning and expression of megakaryocyte potentiating factor. Stem Cells 1996;14 Suppl 1:62–74.

4. Hellstrom I, Raycraft J, Kanan S, et al. Mesothelin variant 1 is released from tumor cells as a diagnostic marker. Cancer Epidemiol Biomarkers Prev 2006;15:1014–20.

5. Chowdhury PS, Viner JL, Beers R, Pastan I. Isolation of a high-affinity stable single-chain Fv specific for mesothelin from DNA-immunized mice by phage display and construction of a recombinant immunotoxin with anti-tumor activity. Proc Natl Acad Sci U S A 1998;95:669–74.

6. Gubbels JA, Belisle J, Onda M, et al. Mesothelin-MUC16 binding is a high affinity, N-glycan dependent interaction that facilitates peritoneal metastasis of ovarian tumors. Mol Cancer 2006;5:50.

7. Rump A, Morikawa Y, Tanaka M, et al. Binding of ovarian cancer antigen CA125/MUC16 to mesothelin mediates cell adhesion. J Biol Chem 2004;279:9190–8.

8. Bera TK, Pastan I. Mesothelin is not required for normal mouse development or reproduction. Mol Cell Biol 2000;20:2902–6.

9. Frierson HF, Jr., Moskaluk CA, Powell SM, et al. Large-scale molecular and tissue microarray analysis of mesothelin expression in common human carcinomas. Hum Pathol 2003;34:605–9.

10. Scholler N, Fu N, Yang Y, et al. Soluble member(s) of the mesothelin/megakaryocyte potentiating factor family are detectable in sera from patients with ovarian carcinoma. Proc Natl Acad Sci U S A 1999;96:11531–6.

11. Kelsey JL, Gammon MD, John EM. Reproductive factors and breast cancer. Epidemiol Rev 1993;15:36–47.

12. MacMahon B, Cole P, Lin TM, et al. Age at first birth and breast cancer risk. Bull World Health Organ 1970;43:209–21.

13. Thordarson G, Jin E, Guzman RC, Swanson SM, Nandi S, Talamantes F. Refractoriness to mammary tumorigenesis in parous rats: is it caused by persistent changes in the hormonal environment or permanent biochemical alterations in the mammary epithelia? Carcinogenesis 1995;16:2847–53.

14. Yang J, Yoshizawa K, Nandi S, Tsubura A. Protective effects of pregnancy and lactation against *N*-methyl-*N*-nitrosourea-induced mammary carcinomas in female Lewis rats. Carcinogenesis 1999;20:623–8.

15. Uehara N, Unami A, Kiyozuka Y, Shikata N, Oishi Y, Tsubura A. Parous mammary glands exhibit distinct alterations in gene expression and proliferation responsiveness to carcinogenic stimuli in Lewis rats. Oncol Rep 2006;15:903–11.

16. Hino O, Fukuda T, Satake N, et al. TSC2 gene mutant (Eker) rat model of a Mendelian dominantly inherited cancer. Prog Exp Tumor Res 1999;35:95–108.

Published OnlineFirst February 1, 2008; DOI: 10.1158/1541-7786.MCR-07-0254

17. Prieve MG, Moon RT. Stromelysin-1 and mesothelin are differentially regulated by Wnt-5a and Wnt-1 in C57mg mouse mammary epithelial cells. BMC Dev Biol 2003;3:2.

18. Yamashita Y, Yokoyama M, Kobayashi E, Takai S, Hino O. Mapping and determination of the cDNA sequence of the Erc gene preferentially expressed in renal cell carcinoma in the Tsc2 gene mutant (Eker) rat model. Biochem Biophys Res Commun 2000;275:134–40.

19. Gordon LA, Mulligan KT, Maxwell-Jones H, Adams M, Walker RA, Jones JL. Breast cell invasive potential relates to the myoepithelial phenotype. Int J Cancer 2003;106:8–16.

20. Soule HD, Vazguez J, Long A, Albert S, Brennan M. A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 1973;51:1409–16.

21. Keydar I, Chen L, Karby S, et al. Establishment and characterization of a cell line of human breast carcinoma origin. Eur J Cancer 1979;15:659–70.

22. Cailleau R, Young R, Olive M, Reeves WJ, Jr. Breast tumor cell lines from pleural effusions. J Natl Cancer Inst 1974;53:661–74.

23. Fukazawa H, Noguchi K, Murakami Y, Uehara Y. Mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) inhibitors restore anoikis sensitivity in human breast cancer cell lines with a constitutively activated extracellular-regulated kinase (ERK) pathway. Mol Cancer Ther 2002;1:303–9.

24. Rytomaa M, Martins LM, Downward J. Involvement of FADD and caspase-8 signalling in detachment-induced apoptosis. Curr Biol 1999;9:1043–6.

25. Fukazawa H, Noguchi K, Masumi A, Murakami Y, Uehara Y. BimEL is an important determinant for induction of anoikis sensitivity by mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitors. Mol Cancer Ther 2004;3:1281–8.

26. Reginato MJ, Mills KR, Paulus JK, et al. Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. Nat Cell Biol 2003;5:733–40.

27. Yang JM, O'Neill P, Jin W, et al. Extracellular matrix metalloproteinase inducer (CD147) confers resistance of breast cancer cells to Anoikis through inhibition of Bim. J Biol Chem 2006;281:9719–27.

28. Ley R, Balmanno K, Hadfield K, Weston C, Cook SJ. Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim. J Biol Chem 2003;278:18811–6.

29. Luciano F, Jacquel A, Colosetti P, et al. Phosphorylation of Bim-EL by Erk1/2 on serine 69 promotes its degradation via the proteasome pathway and regulates its proapoptotic function. Oncogene 2003;22:6785–93.

30. Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol 1994;124:619–26.

31. Frisch SM, Screaton RA. Anoikis mechanisms. Curr Opin Cell Biol 2001;13:555–62.

32. Valentijn AJ, Zouq N, Gilmore AP. Anoikis. Biochem Soc Trans 2004;32:421–5.

33. Eckert LB, Repasky GA, Ulku AS, et al. Involvement of Ras activation in human breast cancer cell signaling, invasion, and anoikis. Cancer Res 2004;64:4585–92.

34. Bharadwaj S, Thanawala R, Bon G, Falcioni R, Prasad GL. Resensitization of breast cancer cells to anoikis by tropomyosin-1: role of Rho kinase-dependent cytoskeleton and adhesion. Oncogene 2005;24:8291–303.

35. Emberley ED, Niu Y, Curtis L, et al. The S100A7-c-Jun activation domain binding protein 1 pathway enhances prosurvival pathways in breast cancer. Cancer Res 2005;65:5696–702.

36. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2002;2:647–56.

37. Marani M, Hancock D, Lopes R, Tenev T, Downward J, Lemoine NR. Role of Bim in the survival pathway induced by Raf in epithelial cells. Oncogene 2004;23:2431–41.

38. Cory S, Huang DC, Adams JM. The Bcl-2 family: roles in cell survival and oncogenesis. Oncogene 2003;22:8590–607.

39. Reddig PJ, Juliano RL. Clinging to life: cell to matrix adhesion and cell survival. Cancer Metastasis Rev 2005;24:425–39.

40. Pages G, Lenormand P, L'Allemain G, Chambard JC, Meloche S, Pouyssegur J. Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation. Proc Natl Acad Sci U S A 1993;90:8319–23.

41. Alahari SK, Reddig PJ, Juliano RL. Biological aspects of signal transduction by cell adhesion receptors. Int Rev Cytol 2002;220:145–84.

42. Attwell S, Roskelley C, Dedhar S. The integrin-linked kinase (ILK) suppresses anoikis. Oncogene 2000;19:3811–5.

43. Ho M, Onda M, Wang QC, Hassan R, Pastan I, Lively MO. Mesothelin is shed from tumor cells. Cancer Epidemiol Biomarkers Prev 2006;15:1751.

AACR American Association for Cancer Research

Molecular Cancer Research

Mesothelin Promotes Anchorage-Independent Growth and Prevents Anoikis via Extracellular Signal-Regulated Kinase Signaling Pathway in Human Breast Cancer Cells

Norihisa Uehara, Yoichiro Matsuoka and Airo Tsubura

Mol Cancer Res 2008;6:186-193. Published OnlineFirst February 1, 2008.