# Autocrine Motility Factor Signaling Enhances Pancreatic Cancer Metastasis

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## ABSTRACT

***Purpose:*** **Autocrine motility factor (AMF)/phosphoglucose isomerase (PGI) is a ubiquitous cytosolic enzyme that plays a key role in glycolysis. AMF/PGI is also a multifunctional protein that acts in the extracellular milieu as a potent mitogen/cytokine. Increased expression of AMF/PGI and its receptor has been found in a wide spectrum of malignancies and is associated with cancer progression and metastasis. Recent studies indicated that AMF is induced by hypoxia and enhances the random motility of pancreatic cancer cells. In the present study, the role and regulation of AMF in the growth and metastasis of pancreatic cancer cells were determined.**

***Experimental Design:*** **In this study, we assessed whether overexpression of AMF in human pancreatic cancer cells enhances the liver metastasis using an orthotopic mouse tumor model. We also investigated the intracellular signal transduction pathways of AMF in human pancreatic cancer cell lines.**

***Results:*** **Overexpression of AMF stimulated *in vitro* invasion of MIA PaCa-2 cells. *In vivo*, after orthotopic implantation into the pancreas of nude mice, parental and empty vector-transfected MIA PaCa-2 cells produced locally relatively small tumors with no evidence of liver metastasis, whereas AMF-transfected MIA PaCa-2 cells produced the large tumors and liver metastases. In addition, over-expression of AMF leads to down-regulation of E-cadherin expression associated with the up-regulation of the zinc-finger transcription factor SNAIL expression.**

***Conclusions:*** **The data submitted here show that AMF expression significantly contributes to the aggressive phenotype of human pancreatic cancer and thus may provide a novel prognostic and therapeutic target.**

## INTRODUCTION

Pancreatic cancer is the fourth leading cause of death because of malignant disease among both men and women in Western countries (1). Pancreatic cancer is usually unresectable at the time of diagnosis because of metastasis or local extension, particularly to the mesenteric vasculature. Most recent series from institutions that specialize in treating pancreatic cancer report mortality rates <5%, with the majority of patients dying of metastatic cancer recurrence (2). More recently, gemcitabine has been used, although life expectancy remains short, and morbidity remains high (3). Clearly, more effective modalities of treatment for advanced pancreatic cancer are needed.

The progression and metastasis of cancer are controlled by extracellular growth factors and cytokines. PGI (EC 5.3.1.9) is a ubiquitous cytosolic enzyme that catalyzes the second step in glycolysis (4). Molecular cloning and sequencing have identified PGI as a motility factor: *i.e.*, autocrine motility factor (AMF; ref. 5), also known as and neuroleukin (6) or maturation factors (7). AMF/PGI is also a multifunctional cytokine that exhibits multifunctional growth factor-like activity via a unique cognate 78 kDa (gp78) seven-transmembrane glycoprotein receptor (autocrine motility factor receptor, AMFR; ref. 8). We have shown that the overexpression of AMF/PGI enhances cell proliferation together with up-regulation of cyclin/cyclin-dependent kinase activities and down-regulation of $p27^{Kip1}$ (9). Overexpression of AMF/PGI and AMFR has been found in a wide spectrum of malignancies and is associated with cancer progression, metastasis (10–12), and angiogenesis (13, 14).

Pancreatic cancer is further characterized by a hypoxic microenvironment within the solid tumor mass (15), a condition activates the hypoxia-inducible factor-1 transcription factor which transcriptionally up-regulates vascular endothelial growth factor (16). Niizeki *et al.*, (17) has shown that that hypoxia enhanced the expression of AMF in various cancer cells and also enhanced the random motility of pancreatic cancer cells (17). However, the expression of AMF in pancreatic cancer has not been characterized. E-cadherin is a cell-to-cell adhesion molecule that participates in homotypic, calcium-dependent interactions to form the epithelial adherens junction. Inactivation of E-cadherin contributes to reduction of cell-to-cell adhesiveness, followed by loss of cell polarity and destruction of histologic structure, and it promotes detachment of tumor cells from the primary lesion (18). Reduced expression of E-cadherin has been reported to correlate with distant metastasis, high-grade, and advanced stage in many types of cancers, including pancreatic cancer (19). In this study, therefore, we examined the effect of AMF on the change in expression of E-cadherin in MIA PaCa-2 cells and its effect on SNAIL, a zinc-finger transcription factor repressing E-cadherin (20–22). We also investigated the

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intracellular signal transduction pathways of AMF in human pancreatic cancer cell lines and have assessed the role of AMF in liver metastasis of human pancreatic cancer cells using an orthotopic mouse tumor model.

## MATERIALS AND METHODS

**Materials.** The following antibodies were used: anti-E-cadherin (PharMingen, San Diego, CA), anti-CD31 (Transduction Laboratories, Lexington, KY), antiactin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and antimouse and antirabbit secondary antibodies (Amersham Biosciences Corp, Piscataway, NJ). Anti-AMF and anti-AMFR were described previously (23, 24). We purified Anti-AMF IgG and preimmune IgG from serum using ImmunoPure (G) IgG according to the manufacturer's instructions (Pierce, Rockford, IL).

**Cell Culture and Transfection.** Human pancreatic cancer cell line (MIA PaCa-2) and human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (Manassas, VA). MIA PaCa-2 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), essential and nonessential amino acids, vitamins, and penicillin/streptomycin. HUVECs were cultured in Ham's F12K medium (Irvine Scientific, Irvine, CA) supplemented with 100 μg/mL heparin (Sigma Chemical Co., St. Louis, MO), 50 μg/mL endothelial cell growth supplement (Collaborative Biomedical Products, Bedford, MA), and 10% FBS. The cells were maintained in a humidified chamber with 95% air and 5% $CO_2$ at 37°C.

The full-length human AMF cDNA was generated by PCR amplification (24). The PCR product was ligated into a mammalian expression vector pcDNA3.1 zeo (Invitrogen, Carlsbad, CA). According to the manufacturer's instructions, LipofectAMINE 2000 (Life Technologies, Inc., Gaithersburg, MD) was used to transfect parental MIA PaCa-2 cells with AMF cDNA. Isolation of single clones of the stable transfectants was accomplished by adding 750 μg/mL of Zeocin (Invitrogen) to the culture medium. The MIA PaCa-2 cell line, stably transfected with pcDNA3.1 zeo or pcDNA3.1 zeo-AMF, was designated MIA-zeo or MIA-AMF, respectively. All experiments were repeated at least three times, and results were confirmed by both clonal cell lines and the pooled cell population.

**Northern Blot Analysis and Reverse Transcription-PCR (RT-PCR).** Trizol reagent (Invitrogen) was used to isolate total cellular RNA, and $^{32}$P-labeled AMF cDNA probe (24) was used to analyze 20 μg of RNA via Northern hybridization. RT-PCR analysis was done as described (9). Primers were synthesized to encompass a specific segment of the cDNA sequence of *E-cadherin* (forward, 5′-TCCATTTCTTGGTCTACGCC-3′ and reverse, 5′-CACCTTCAGCCAACCTGTTT-3′), *Snail* (forward, 5′-AATCGGAAGCCTAACTACAAG-3′ and reverse, 5′-AGGAAGAGACTGAAGTAGAG-3′), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*, forward, 5′-ACGACCACTTTGTCAAGCTC-3′ and reverse, 5′-TCACAGTTGCCATGTAGACC-3′). The corresponding cDNA fragments were denatured at 94°C for 30 seconds, annealed at 58°C for 1 minute, and extended at 72°C for 1 minute. After 35 cycles of amplification, the PCR products were electrophoresed on a 2% agarose gel containing 10 μg/mL ethidium bromides, and intensity of the bands was measured by a Kodak Digital Science Image System (Rochester, NY). Gene expression was presented by the relative yield of PCR product from the target sequence to that from the *GAPDH* gene.

**Western Blot Analysis.** The cells were lysed in lysis buffer (50 mmol/L HEPES (pH 7.9), 0.1% NP40, 10% glycerol, 1 mmol/L DTT, 1% sodium deoxycholate, 250 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, and 0.1 mg/mL leupeptin) at 4°C. Cell lysates containing equal amounts of protein were separated by SDS-PAGE, and transferred to a polyvinylidene fluoride membrane (MSI, Westborough, MA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBS-T) incubated with primary antibody for 2 hours, washed 3 times during 15 minutes in TBS-T, incubated with the secondary horseradish peroxidase-conjugated antibody (ZYMED, San Francisco, CA) for 1 hour, and finally washed three times. The horseradish peroxidase activity was detected by an incubation of the membrane with enhanced chemiluminescence reagent (Amersham, Arlington Heights, IL). A Kodak imaging system determined density of the bands.

**Cell Proliferation Assays.** Cell proliferation assays were done by seeding cells at a density of $1 \times 10^5$ cells/well in 6-well plates. Cells were fed DMEM with 10% FBS every other day and counted daily.

**Invasion Assay.** The invasive activity of cells was assayed in transwell cell culture chambers (Corning Costar Co., Cambridge, MA). Polycarbonate filters with 8-μm pore size were coated with Matrigel (1 mg/mL, Collaborative Biomedical Products, Bedford, MA) to form a matrix barrier. Cells were resuspended to a concentration of $1 \times 10^6$/mL in DMEM with 0.5% FBS. The cell suspension (100 μL) was added to the upper compartment of the chamber, and incubated with DMEM with 10% FBS in the lower compartment for 24 hours at 37°C. The filters were fixed with 4% paraformaldehyde and stained with Hema 3 (Fisher Scientific, Pittsburgh, PA). The cells on the upper surface of the filters were removed by wiping with cotton swabs. The cells that had invaded through Matrigel and the filter to the lower surface were counted. Each assay was done in triplicate. In some experiments, anti-AMF IgG (50 μg/mL) or preimmune IgG (50 μg/mL) was incubated in the medium.

**HUVEC Adhesion Assay.** HUVEC adhesion assay was done as described previously (25). Parental MIA PaCa-2, MIA-zeo, and MIA-AMF cells were suspended at a concentration of $3 \times 10^6$ cells/mL in serum-free medium containing 1% BSA and radio labeled with 5 μCi of $Na^{51}CrO_4$ (DuPont NEN Research Products, Boston, MA) for 2 hours at 37°C. At the end of the incubation, the cell suspensions were washed extensively and plated in quadruplicate in 16-mm Costar culture dishes (Corning Costar) containing HUVEC monolayers. After 2 hours, the cells were washed gently and thoroughly with PBS, and the attached cells were treated with 1% Triton X-100. The lysates was collected, and the radioactivity in each well was measured with a gamma counter. The number of attached cells was calculated from the specific radioactivity of the labeled cells. The adhesion of parental MIA PaCa-2 cells to HUVECs in control experiments was given the value of 100%; the percentage of adhesion of MIA-zeo and MIA-AMF cells were calculated accordingly.

**Orthotopic Tumor Model.** Female athymic nude mice were housed under specific pathogen-free conditions and used at 6 weeks of age. For the implantation, a median incision was made in the abdomen, and the peritoneum was separated from the abdominal wall and opened. The pancreas was carefully exposed, and $2 \times 10^6$ tumor cells in PBS (total volume of 20 μL) were slowly injected into the capsule of pancreatic body. The pancreas was then returned into the peritoneal cavity, and the abdominal wall and the skin were closed in two layers by 6–0 surgical sutures. All of the procedures were done with standard surgical loupes. The mice tolerated the procedure well without any complications. The volume ($V$) of the xenograft was calculated by the following formula: $V = \pi/6(a \times b \times c)$, where $a$, $b$, and $c$ represent the length, width, and height of the mass. The mean values and the SDs of the tumor volumes were calculated. Ten mice were inoculated with each type of cell in two separate experiments.

**Histopathologic and Immunohistochemical Study.** The tumors were fixed in 10% phosphate-buffered formalin, and paraffin-embedded 4 μm-thick sections were prepared. Slides were stained with H&E stain according to standard laboratory protocols. We did immunohistochemical study using Vectastain Elite ABC kit according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA).

**Clinical Samples.** Pancreatic cancer tissues were obtained from 13 patients (7 women and 6 men) undergoing initial surgical resection, without any prior therapy. The median age of the pancreatic carcinoma patients was 65 years (range, 48–77 years). According to the TNM classification and Histopathologic grading system (26), there were one stage IIA and 12 stage IIB tumors. Control tissue samples (normal pancreas and pancreatitis) were obtained from 11 individuals (4 female and 7 male) who had undergone pancreas resection. Eight normal pancreas tissue specimens were derived from four patients with biliary tract cancer and four patients with gastric cancer with histologically normal pancreas removed distant from the tumor. Chronic pancreatits tissues were obtained from three male patients undergoing a pancreatectomy because of chronic pancreatitis. The median age of the control group was 64 years, with a range of 48 to 75 years. Formalin-fixed, paraffin-embedded tissue sections were subjected to immunostaining by anti-AMF IgG and anti-Ecadherin, as describe above. To ensure specificity of the primary antibodies, we incubated consecutive sections either in the absence of the primary antibody or with a nonimmunized mouse or rabbit IgG antibody. In these cases, no immunostaining was detected.

**Statistical Analysis.** A Student's $t$ test was used to determine statistical analyses. $P < 0.05$ was considered significant.

## RESULTS

**Constitutive Overexpression of AMF in MIA PaCa-2 Transfectants.** After transfection, human AMF cDNA three stably transfected cell clones exhibiting high-level expression of AMF in MIA PaCa-2 cells were selected and established (MIA-AMF1, -AMF2, and -AMF3, three individual clones). As shown in Fig. 1*A*, the AMF ratios of each clone were 5 to 6 compared with the empty vector-transfected control (MIA-zeo). Consistent with mRNA expression, MIA-AMF cells secreted an elevated amount of AMF, whereas MIA PaCa-2 and MIA-zeo cells secreted ~3-fold less protein (Fig. 1*A*). Of note, no differences in expression of AMFR could be observed among the AMF transfectants and vector-only transfected cells (Fig. 1*A*). Previously, it was shown that AMF promotes growth of NIH/3T3 cells (27). Thus, we tested whether overexpression of AMF on MIA PaCa-2 human pancreatic cancer cells will affect their *in vitro* growth properties. No statistical significance in the growth rate among the cell variants could be detected (Fig. 1*B*). Although AMF might be a minimal autocrine growth factor for MIA PaCa-2 cells *in vitro*, this newly acquired proliferation capability of AMF-transfected NIH/3T3 cells can be attribute to contact inhibition. Ectopic AMF expression induced NIH/3T3 cell transformation. An important growth variable that distinguishes between untransformed NIH/3T3 cells and transformed cells is the loss of contact inhibition. Parental MIA PaCa-2 cells exhibited a disorganized pattern of growth, indicating a loss of contact inhibition in itself. Although AMF increased the invasive ability of MIA-AMF cells 1.5- to 2-fold as compared with control cells (parental MIA PaCa-3 and MIA-zeo) at 24 hours (Fig. 1*C*), this invasive ability was specifically inhibited by anti-AMF IgG (50 μg/mL) but not by preimmune rabbit IgG (Fig. 1*C*).

**AMF Increases Adhesion to HUVEC.** To examine the possible effects of AMF on cancer cell adhesion to HUVECs, a monolayer cell adhesion assay was carried as described in the Material and Method section. As shown in Fig. 1*D*, AMF-transfected MIA PaCa-2 cells adhered significantly more to HUVECs than the parental or empty vector-transfected MIA PaCa-2 cells. This adhesion of tumor cells to HUVECs was inhibited by anti-AMF IgG but not by preimmune IgG (Fig. 1*D*).

**The Effect of AMF Expression on the *In vivo* Growth of Human Pancreatic Cancer Cells.** To determine tumorigenicity and metastatic potential of the following cell, parental MIA PaCa-2, MIA-zeo, and MIA-AMF cells ($2 \times 10^6$ cells/mouse) were orthotopically injected into the pancreas of nude mice ($n = 10$ for each variant). As described previously (28), orthotopically implanted control cells (parental MIA PaCa-2 and MIA-zeo) formed small tumors (Fig. 2*A*), and in sharp contrast, the AMF-transfected cells produced larger and more aggressive tumors (Fig. 2*A*). The metastatic potential of the cells was determined 1 month after orthotopic implantation of tumor cells. MIA-AMF cells produced spontaneous liver metastases (Fig. 2*A*), whereas the parental MIA PaCa-2 and MIA-zeo cells were never metastatic. These data show that increased expression of AMF leads to enhancement of tumor formation and metastasis of human pancreatic cancer cells (Table 1). Parental MIA PaCa-2 and MIA-zeo tumors did not invade surrounding tissues and showed localized tumor growth (Fig. 2*B*), whereas MIA-AMF cells were focally invading surrounding pancreatic tissue (Fig. 2*B*). Previous studies have indicated that AMF may act as a direct or an indirect angiogenic factor (13, 14). Thus, we processed the primary tumors for immunohistochemical analyses of vascular formation using anti-CD31 as a vessel marker. As shown in Fig. 2*B*, all AMF-transfected tumors were highly vascular, in contrast, to the limited vascularity of the tumors formed by control cells (parental MIA PaCa-2 and MIA-zeo;

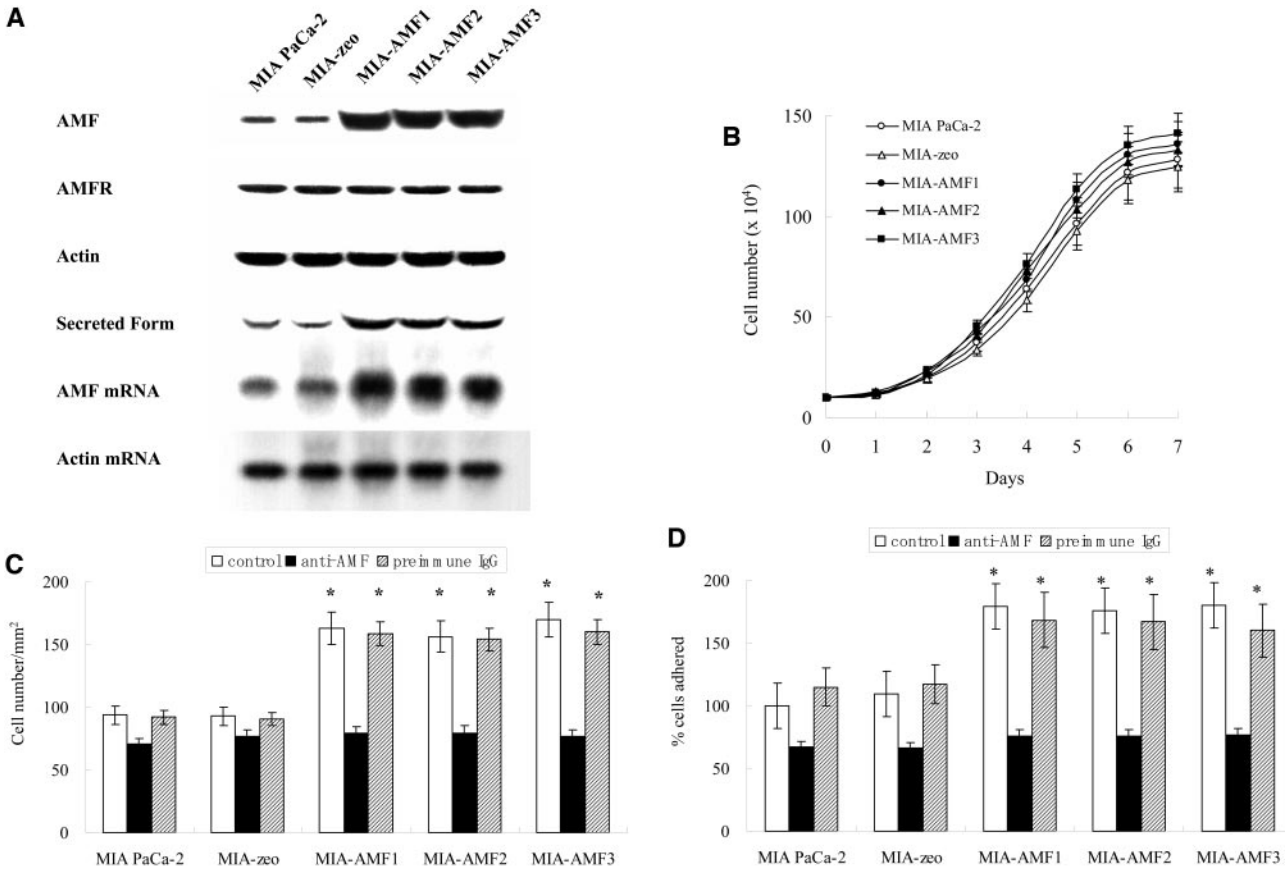


*Fig. 1* *A*, AMF expression in pancreatic cancer cells transfected with AMF expression vectors. The parental MIA PaCa-2 cells were stably transfected with empty vector (MIA-zeo), and AMF expression vector (MIA-AMF1, MIA-AMF2, and MIA-AMF3, three individual clones) and cell lysates from these cells were subjected to Western blot analysis with anti-AMF antibody (*top row*) or anti-AMFR antibody (*second row*). The *third row* was probed with antiactin antibody as a control. The secretion of AMF was analyzed by Western blotting of 50 μg of protein from conditioned medium (*fourth row*) and probed with anti-AMF antibody. Different protein preparations were used to do the experiment in triplicate. Northern blot analysis. Twenty μg of RNA were electrophoresed through a 1% denaturing formaldehyde-agarose gel, transferred to nylon membrane, and hybridized to the $^{32}$P-labeled AMF cDNA (*fifth row*). $^{32}$P-labeled β-actin was used as a loading control (*bottom row*). *B*, growth properties of AMF-transfected MIA PaCa-2 cells. Cells were grown in medium containing 10% FBS, and cell numbers were then determined. Each *bar* represents the mean of triplicate determinations ± SD Similar results were obtained in three independent experiments. ○, parental MIA PaCa-2; △, empty vector-transfected MIA PaCa-2; ●, AMF-overexpressing MIA PaCa-2 clone 1; ▲, AMF-overexpressing MIA PaCa-2 clone 2; ■, AMF-overexpressing MIA PaCa-2 clone 3. *C*, invasive ability of AMF-transfected MIA PaCa-2 cells. Two-hundred μl of a single-cell suspension ($1 \times 10^6$ cells/mL) of cells were placed in the upper wells of individual transwell inserts containing 8-μm pore polycarbonate membranes precoated with Matrigel. Cells were allowed to invade for 24 hours at 37°C, and then they were fixed and stained with Hema-3. Cells on the upper surface were removed with a cotton swab, and the cells that migrated to the lower side of the membrane were mounted on a microscope slide and counted under a light microscope at ×200 magnification. *In vitro* invasive ability was significantly greater in AMF-transfected cells when compared with parental and empty vector-transfected MIA PaCa-2 cells at 24 hours (□). Invasive capacity was inhibited by anti-AMF IgG (50 μg/mL, ■) but not preimmune IgG (50 μg/mL, ▨). Data shown are the mean values from three triplicate experiments for each group; *bars*, ±SE. ∗, significant difference when compared with control ($P < 005$). *D*, adhesion of tumor cells to HUVECs. Parental, empty vector-transfected, AMF-transfected MIA PaCa-2 cells were labeled with $Na^{51}CrO_4$ and incubated with HUVECs. After 2 hours, the cells were washed, lysed, and counted by scintillation counter (□). Controls were given a value of 100%, and the other values were calculated accordingly. Adhesive capacity was inhibited by anti-AMF IgG (50 μg/mL, ■) but not preimmune IgG (50 μg/mL, ▨). Each value represents a mean of three readings. *Error bars* represent 95% confidence intervals. ∗, $P < 0.001$.

Fig. 2*B*). By counting vessel number, we determined that there was a 250% increase in vascular density of tumors overexpressing AMF compared with all control tumors.

**AMF Signal Transduction Pathways of AMF in Human Pancreatic Cancer.** One characteristic of the AMF-transfected cells is their ability to invade tissues and liver metastasis. Thus, we examined the molecular mechanisms involved in AMF invasion-motility signaling pathway, because it is an important event in the initial metastatic cascade. Loss of E-cadherin expression is considered as a central event in cancer cell invasion and motility, as reduction of cell-to-cell contact facilitates their ability to invade (18, 19). In the AMF-transfected MIA PaCa-2 cell lines, the expression of E-cadherin protein and mRNA was reduced by 30 to 40% of the levels found in control cells as determined by densitometric tracing analysis (Fig. 3*A*). RT-PCR analysis of *SNAIL*, a transcriptional repressor of E-cadherin

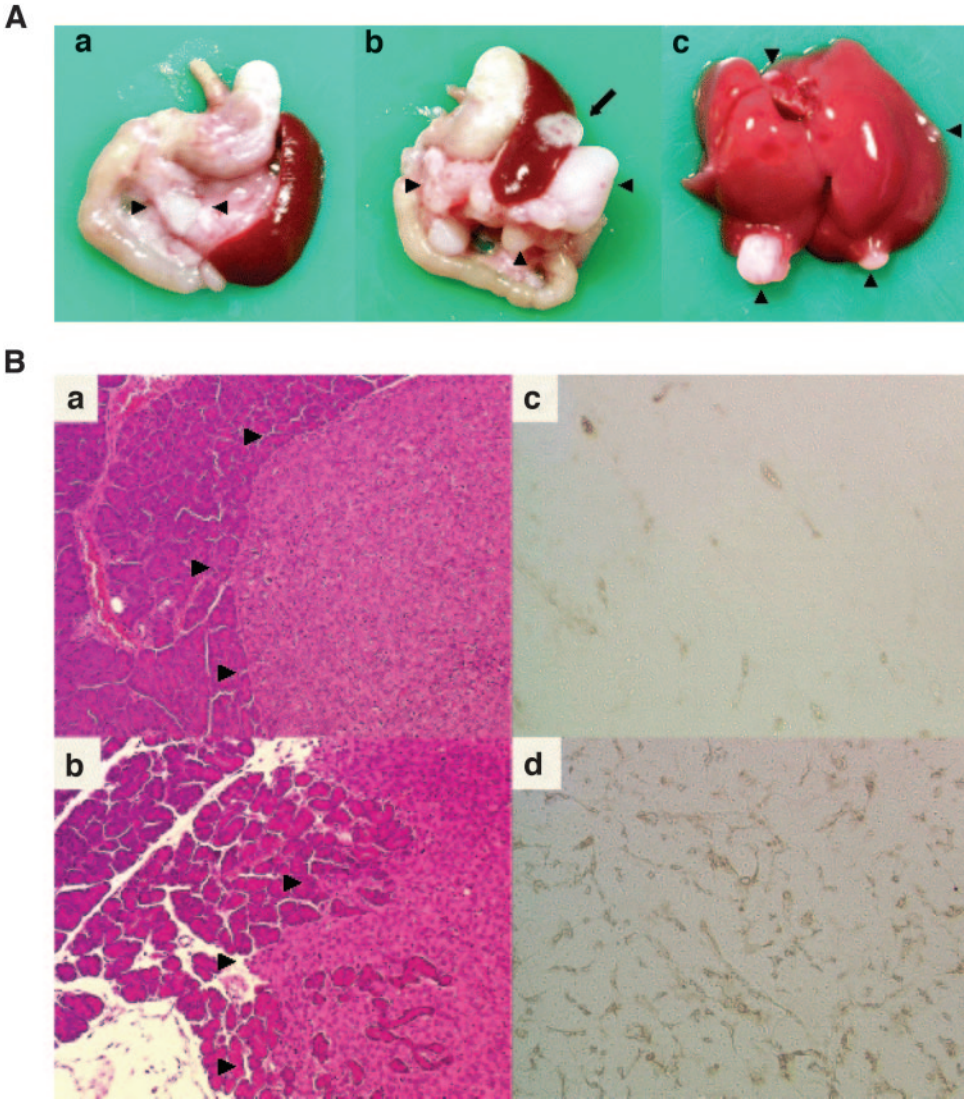


*Fig. 2* *A*, AMF enhanced growth and metastasis *in vivo*. To evaluate the effect of AMF signaling on local and distant metastatic tumor growth, a more clinically relevant orthotopic tumor model was selected. Parental and empty vector-transfected MIA PaCa-2 cells formed small tumors (*a*, *arrow head*), whereas the AMF-transfected cells formed more aggressive tumors (*b*, *arrow head*). AMF-transfected MIA PaCa-2 cells produced spontaneous splenic (*b*, *arrow*) and liver metastases (*c*, *arrow head*), whereas the parental and empty vector-transfected MIA PaCa-2 cells were nonmetastatic in a reproducible manner. *B*, histologic appearance of AMF-overexpressing tumors in nude mice. Tumors were generated by orthotopical inoculation of AMF-transfected MIA PaCa-2 cells as described in "Materials and Methods." Parental and empty vector-transfected MIA PaCa-2 tumors did not invade surrounding tissues and showed localized tumor growth (*a*, *arrow head*), whereas AMF-transfected cells were focally invading surrounding pancreatic tissue (*b*, *arrow head*). We analyzed the formation of neovasculature by immunohistochemical staining (anti-CD31) in AMF-transfected MIA PaCa-2 tumors obtained from the orthotopic pancreatic cancer xenografts. The number of blood vessels was significantly increased in AMF-transfected tumors (*d*) as compared with control tumors (*c*).

revealed a 2.7-fold increase in the mRNA level relative to control (Fig. 3*B*). SNAIL mRNA levels of AMF-transfected MIA PaCa-2 cells reverted to that of control cells by anti-AMF IgG (Fig. 3*D*) but not by preimmune rabbit IgG (Fig. 3*E*).

**Overexpression of AMF in Human Pancreatic Cancer.** Expression of AMF protein was examined in paraffin sections from 13 pancreatic cancer and control patients by immunohistochemistry. Their clinicopathologic characteristics are summarized in Tables 2 and 3. Strong cytoplasmic staining of AMF protein was detected in 11 cancers but was weakly expressed in two cancers and nontumor tissues (Fig. 4). Representative example of AMF and E-cadherin expression of human pancreatic cancer was determined by immunohistochemistry (Fig. 4*A* and *E*; normal pancreatic duct, *B* and *F*; poorly differentiated adenocarcinoma, *C*, *D*, *G*, and *H*; moderately differentiated adenocarcinoma). Strongly positive AMF expression is present in the cytoplasm of cancer cells (Fig. *4B* and *C*) but not normal pancreatic duct cells (Fig. 4*A*). All of the normal epithelial cells

*Table 1* Effect of AMF overexpression on pancreatic tumors in nude mouse

| Cell line | Orthotopic Pancreatic Tumor | | Metastasis (incidence) | |
|---|---|---|---|---|
| | Incidence | Volume ($mm^3$) | Liver | Spleen |
| MIA PaCa-2 | 7/10 | 17.8 ± 4.3 | 0/10 | 0/10 |
| MIA-zeo | 8/10 | 23.3 ± 5.3 | 0/10 | 0/10 |
| MIA-AMF1 | 10/10 | 45.0 ± 9.3 * | 7/10 | 7/10 |
| MIA-AMF2 | 10/10 | 36.9 ± 7.1 * | 7/10 | 5/10 |
| MIA-AMF3 | 10/10 | 43.6 ± 8.4 * | 6/10 | 6/10 |

* $P < 0.01$ *versus* MIA PaCa-2 and MIA-zeo.

express E-cadherin strongly on the cell membrane (Fig. 4*E*). E-cadherin expression of tumor cells is negative (Fig. 4*F* and *G*). Expression of E-cadherin is weak and heterogeneous (Fig. 4*H*). Inverse relation is seen between the expression of AMF and E-cadherin.

Relations between AMF expression level and clinicopathologic factors including the prognoses of the patients were not attributable to the limited amount of available clinical sample. All clinical samples were obtained from stage IIA or stage IIB patients because about 90% of patients had surgically unresectable disease at time of diagnosis (2).

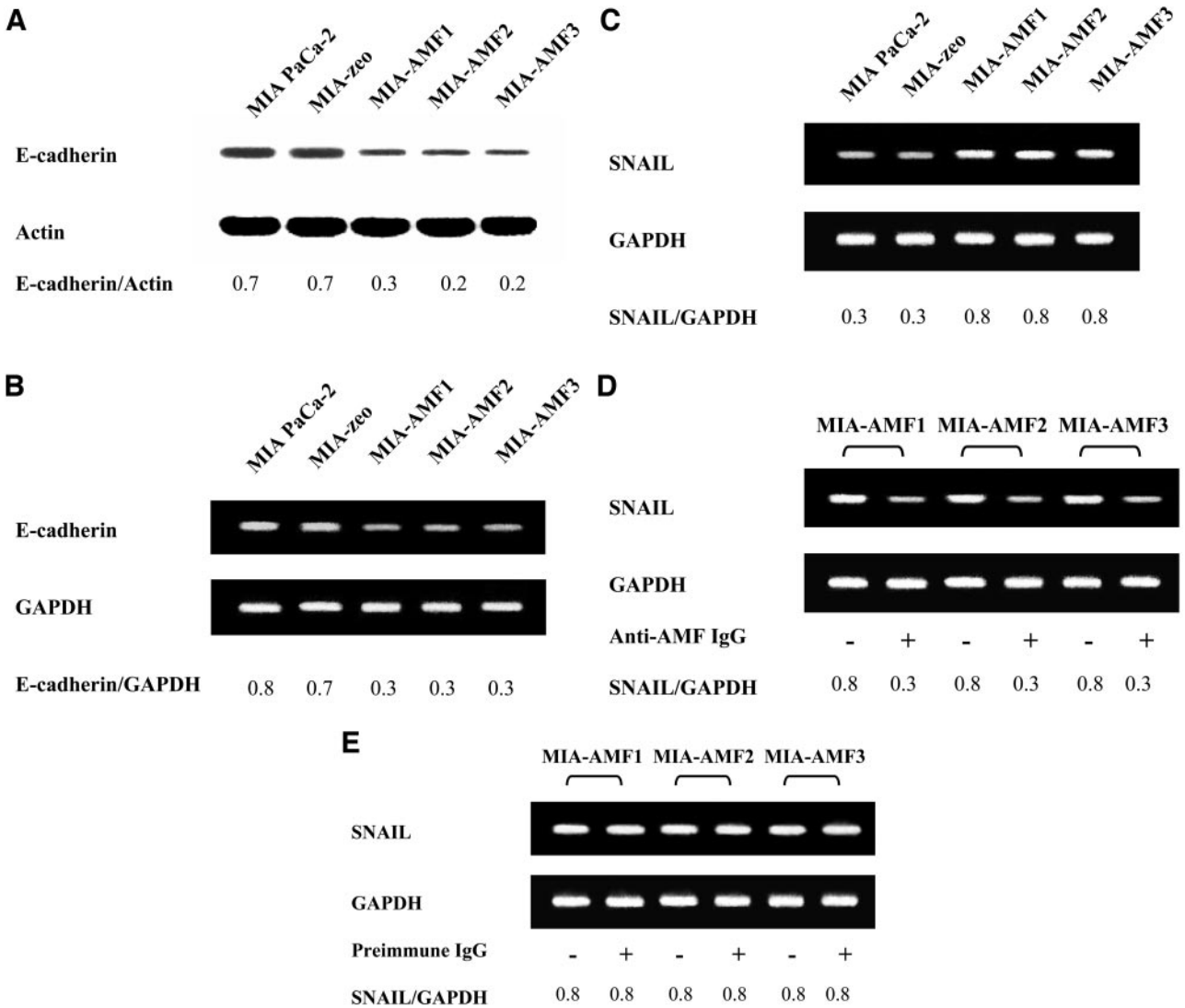


*Fig. 3* Repression of E-cadherin in AMF-overexpressing MIA PaCa-2 cells. *A*, E-cadherin, homotypic cell-to-cell adhesion molecule, expression was analyzed by immunoblotting. Down-regulation of E-cadherin was observed in AMF-transfected MIA PaCa-2 cells compared with control cells. Equal loading was confirmed by immunoblotting the membrane with an antibody to actin (*bottom row*). *B*, RT-PCR analysis of *E-cadherin*, mRNA levels *GAPDH* (*bottom row*) as the internal control. The amount of E-cadherin protein and mRNA in AMF-transfected cells reduced 30 to 40% of the levels found in parental cells by densitometric tracing analysis. *C*, RT-PCR analysis of *SNAIL*, transcriptional repressor of E-cadherin, mRNA levels using *GAPDH* (*bottom row*) as the internal control. Measurement of the expression of *SNAIL* mRNA revealed ratios of 2.7 relative to control cells by densitometric tracing analysis. *D*, *SNAIL* mRNA levels of AMF-transfected MIA PaCa-2 cells reverted to that of control cells by anti-AMF IgG but not by preimmune IgG (*E*).

*Table 2* Characteristics of patients and pancreatic cancers

| Case | Age | Sex | Site | G * | T * | N * | M * | Stage * | AMF Stain |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 69 | F | Head | $G_3$ | $T_2$ | $N_1$ | $M_0$ | IIB | Strong |
| 2 | 73 | M | Head | $G_2$ | $T_2$ | $N_1$ | $M_0$ | IIB | Strong |
| 3 | 59 | M | Head | $G_2$ | $T_2$ | $N_1$ | $M_0$ | IIB | Weak |
| 4 | 62 | M | Tail | $G_3$ | $T_3$ | $N_1$ | $M_0$ | IIB | Strong |
| 5 | 57 | M | Body | $G_2$ | $T_2$ | $N_1$ | $M_0$ | IIB | Strong |
| 6 | 60 | F | Head | $G_2$ | $T_3$ | $N_0$ | $M_0$ | IIA | Weak |
| 7 | 66 | F | Head | $G_2$ | $T_3$ | $N_1$ | $M_0$ | IIB | Strong |
| 8 | 77 | F | Head | $G_2$ | $T_2$ | $N_1$ | $M_0$ | IIB | Strong |
| 9 | 73 | M | Head | $G_1$ | $T_3$ | $N_1$ | $M_0$ | IIB | Strong |
| 10 | 48 | F | Head | $G_1$ | $T_2$ | $N_1$ | $M_0$ | IIB | Strong |
| 11 | 61 | M | Head | $G_1$ | $T_2$ | $N_1$ | $M_0$ | IIB | Strong |
| 12 | 68 | F | Head | $G_2$ | $T_3$ | $N_1$ | $M_0$ | IIB | Strong |
| 13 | 72 | F | Head | $G_2$ | $T_3$ | $N_1$ | $M_0$ | IIB | Strong |

* Tumor Node Metastasis classification of malignant Tumors (26).

## DISCUSSION

Despite improvements in early diagnosis, surgical techniques, and chemotherapy, the majority of pancreatic cancer patients die from metastasis. The aggressive nature of this disease is related to several abnormalities in growth factors and their receptors in the growing cancer (29). Cancer metastasis is a multistep process involving complex and highly coordinated interactions between tumor cells and a constantly changing host microenvironment (30). Like many other malignant diseases, pancreatic cancer results from the accumulation of acquired genetic mutations. More than 85% of pancreatic cancers have an activating point mutation in the K-*ras* gene at a very early stage of pancreatic cancer development (31). The *p16* tumor-suppressor gene is inactivated in around 95% of pancreatic cancers (32). The second most frequently inactivated tumor-suppressor gene is *TP53* located on chromosome 17p (33), whereas MADH4 (*DPC4* or *SMAD4*) is inactivated in 55% of pancreatic adenocarcinomas (34). Thus, the genetic level, pancreatic cancer is a well-characterized; in contrast, the molecular mechanisms linking the genetic changes to the aggressive nature of this disease remain poorly understood. The biology of pancreatic cancer is thought to be related to abnormalities in growth factors and their receptors, which affect the downstream signal transduction pathways involved in the control of growth and differentiation (33), including the epidermal growth factor family (29), VEGF (35), and cytokines, like interleukin 8 (36). It was also reported that the aberrant expression of these are regulated by hypoxia including AMF expression (17, 37). Human pancreatic cancers exhibit significant hypoxia (38). The adaptation of cancer cells to hypoxia is regulated by hypoxia-inducible factor-1, a key transcription factor that up-regulates a series of genes involved in glycolytic energy metabolism, which improve cell survival and promote the progression of cancers that rely on aerobic glycolysis (39). Hypoxia enhanced the expression of AMF in various cancer cells and enhanced the motility of pancreatic cancer cells (17, 37). AMF was recently shown to act as an angiogenic factor *in vivo* and *in vitro* (13, 14). Here, we show the development of many microvessels in AMF-overexpressing tumors compared with control tumors, which support the survival, growth, and metastasis of these cells. Likewise the increased expression of this glycolytic enzyme probably supports the survival and growth of these cells under hypoxic conditions in the growing tumors (40). Cancer cell energy metabolism deviates significantly from that of normal tissues. Cancer cells maintain high aerobic glycolytic rates and produce high levels of lactate and pyruvate. This phenomenon was first described in cancer more than seven decades ago and is known historically as the Warburg effect (41). Preferential reliance on glycolysis is correlated with disease progression in several types of cancers, and the activities of glycolytic enzymes are consistently and significantly increased in cancer cells (42). The *ras*, *src*, and *myc* oncogenes enhance aerobic glycolysis by increasing the expression of glucose transporters and glycolytic enzyme activ-

*Table 3* Pancreatic cancer patients' characterization

| Control | Age | Sex | Primary disease | Pathological diagnosis | AMF Stain |
|---|---|---|---|---|---|
| 1 | 74 | F | Biliary tract cancer | Normal pancreas | Weak |
| 2 | 69 | F | Biliary tract cancer | Normal pancreas | Weak |
| 3 | 75 | M | Biliary tract cancer | Normal pancreas | Weak |
| 4 | 72 | M | Biliary tract cancer | Normal pancreas | Weak |
| 5 | 65 | F | Gastric cancer | Normal pancreas | Weak |
| 6 | 64 | M | Gastric cancer | Normal pancreas | Weak |
| 7 | 66 | F | Gastric cancer | Normal pancreas | Weak |
| 8 | 65 | M | Gastric cancer | Normal pancreas | Weak |
| 9 | 48 | M | Pancreatitis | Chronic pancreatitis | Weak |
| 10 | 55 | M | Pancreatitis | Chronic pancreatitis | Weak |
| 11 | 53 | M | Pancreatitis | Chronic pancreatitis | Weak |

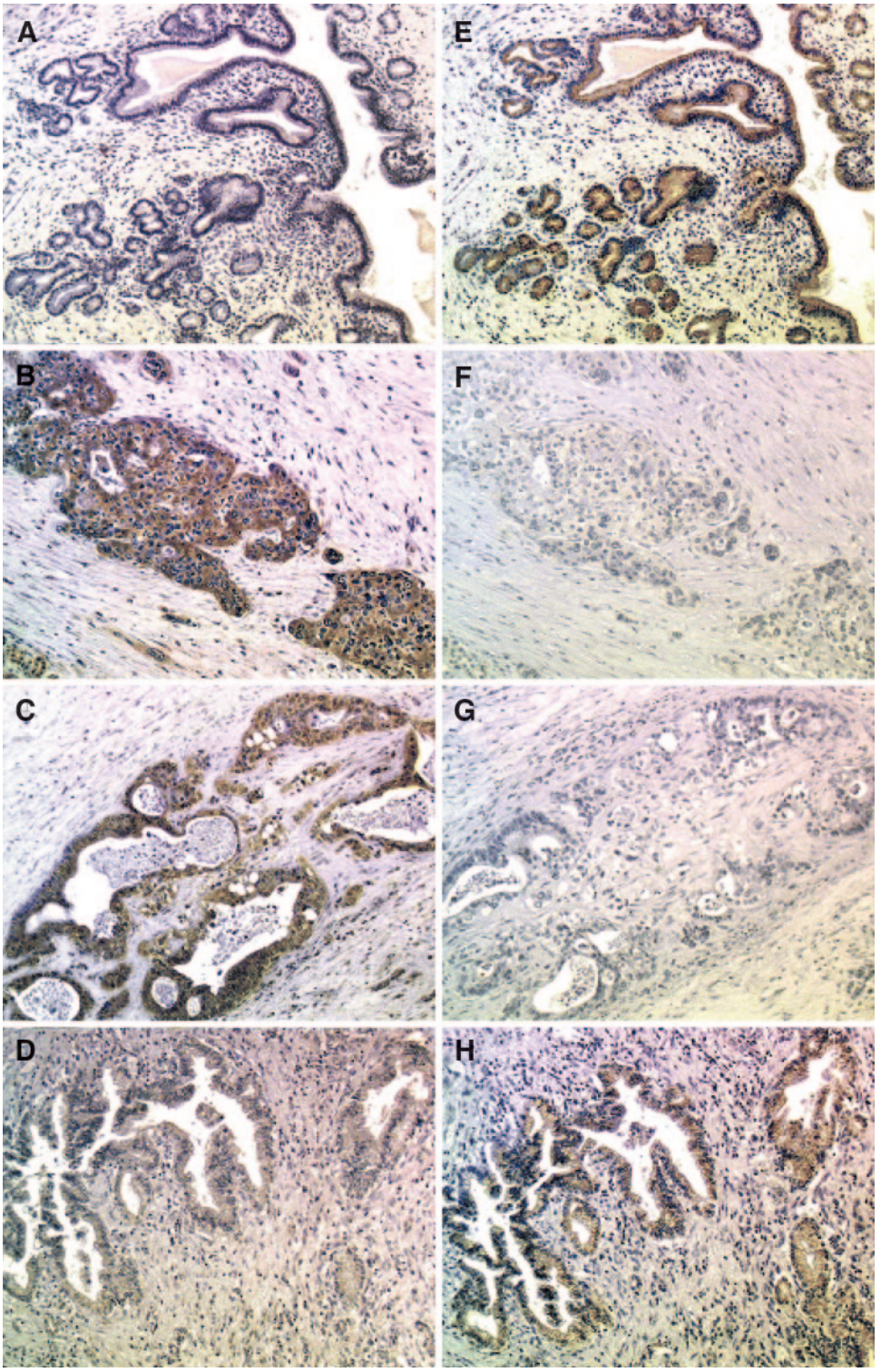


*Fig. 4* Expression of AMF in human pancreatic cancers. Representative example of AMF and E-cadherin expression of human pancreatic cancer determined by immunohistochemistry (*A* and *E*, normal pancreatic duct; *B* and *F*, poorly differentiated adenocarcinoma; *C*, *D*, *G*, and *H*, moderately differentiated adenocarcinoma). Strongly positive AMF expression is present in the cytoplasm of cancer cells (*B* and *C*) but not normal pancreatic duct cells (*A*). Low level of AMF expression is present in the sample of cancer tissue (*D*). All of the normal epithelial cells express E-cadherin strongly on the cell membrane (*E*). E-cadherin expression of tumor cells is negative (*F* and *G*). Expression of E-cadherin is weak and heterogeneous (*H*). Inverse relation is seen between the expression of AMF and E-cadherin.

ities (42–44); in addition, the multifunction of AMF/PGI may contribute to such effects.

Disruption of E-cadherin is considered a key step in progression toward the invasive phase of cancer (45). We show here that overexpression of AMF down-regulates total E-cadherin protein as measured by Western blot. In many types of cancer, E-cadherin is down-regulated without mutation in the gene (18) at the level of its transcription (20–22). Several

transcription factors were implicated in E-cadherin repression including the zinc-finger transcription factor SNAIL that interacts with E-box sequences in the proximal *E-cadherin* promoter (20–22). Because SNAIL was capable of repressing *E-cadherin* promoter activity and E-cadherin expression, we sought to characterize expression of SNAIL in pancreatic cells by RT-PCR. Transcription of *SNAIL* was elevated in AMF-transfected MIA PaCa-2 cells compared with control cells. These may suggest that AMF contributes to tumor progression in part because of inactivation of E-cadherin expression through SNAIL in pancreatic cancer, and we hypothesize that the functional activation of AMF during cancer progression may represent a novel mechanism for down-regulation of E-cadherin expression in invasive tumors. The formation of metastases depends on a series of events, including the extravasation of tumor cells in the capillary bed of secondary organs and the continued survival of those cells. Adhesion of tumor cells to vascular endothelial cells is initiated by the binding of E-selectin on endothelial cells to the carbohydrate ligands on tumor cells. The carbohydrate determinants, sialyl $Le^x$ and sialyl $Le^a$ on tumor cells, serve as ligands for E-selectin in the initial adhesion, followed by the secondary adhesion mediated by integrins (46). Our previous report showed that AMF stimulates integrin-dependent tumor cell adhesion, spreading, and invasion (47). We concluded that AMF regulates adhesive property of AMF-transfected MIA PaCa-2 cells to HUVECs by integrin-mediated adhesion.

Conventional treatment approaches, such as surgery, radiation, chemotherapy, or combinations of these, have had little impact on disease course. Thus, intense interest has focused on the emerging molecular biology of pancreatic cancer. Already various agents are being studied and developed that target signal transduction pathways or nuclear transcription factors. Matrix metalloproteinase inhibitors, farnesyltransferase inhibitors, and tyrosine-kinase inhibitors and monoclonal antibodies against growth factors or their receptors are novel agents that have undergone phase II or III trials (33, 48). Phase III studies of matrix metalloproteinase inhibitors, alone or in combination with gemcitabine (49), and phase III studies of farnesyltransferase inhibitors have produced disappointing results (50). Targeting of epidermal growth factor receptor and its family members with monoclonal antibodies has become possible with the recent introduction of chimeric and humanized antibodies (33, 48). On the other side, clinical trials investigating the activity of epidermal growth factor receptor tyrosine-kinase inhibitors, gefitinib (ZD1839) and erlotinib (OSI-774), in pancreatic cancer are currently ongoing (33, 48). VEGF expression correlates positively with local recurrence, metastatic potential, and overall survival. The VEGF pathway has thus emerged as a therapeutic target in pancreatic cancer. Bevacizumab is a humanized VEGF antibody that can be used safely, either alone or in association with several chemotherapy combinations (33, 48). Additional study will be required to evaluate whether the AMF/PGI could be a therapeutic target candidate.

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