Embigin Is Overexpressed in Pancreatic Ductal Adenocarcinoma and Regulates Cell Motility Through Epithelial to Mesenchymal Transition via the TGF-β Pathway

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Embigin is a member of the immunoglobulin superfamily and encodes a transmembrane glycoprotein. There have been reports of Embigin involvement in neuromuscular junction formation and plasticity; however, the molecular functions of Embigin in other organs are unknown. Our aim was to investigate the possible role of Embigin in pancreatic cancer. In pancreatic ductal adenocarcinoma tissues, Embigin expression was higher than that in normal pancreatic tissues. Immunohistochemical analysis revealed expression of Embigin in pancreatic cancer cells, as well as expression of monocarboxylate transporter 2 (MCT2) in cancer tissues. To gain further insight, we transfected BxPC-3 and HPAC pancreatic cancer cells with siRNA or shRNA targeting Embigin and observed reductions in cell proliferation, migration, invasion, wound healing, and reduced levels of matrix metalloproteinases-2 and -9. Silencing of Embigin increased intracellular L-lactate concentration by 1.5-fold and decreased MCT2 levels at the plasma membrane. Furthermore, Embigin silencing led to a reduced expression of PI3K, GSK3-β, and Snail/Slug. Upon treating BxPC-3 cells with transforming growth factor-β (TGF-β), we observed elevated expression of Snail/Slug, Embigin, and Vimentin; meanwhile, when treating cells with SB-216763, a GSK3-β inhibitor, we noted decreases in GSK3-β, Snail/Slug, and Embigin expression, suggesting that the TGF-β signaling cascade, comprising PI3K, GSK3-β, Snail/Slug, and Embigin signals, mediates epithelial to mesenchymal transition (EMT) in pancreatic cancer cells. These findings indicate the involvement of Embigin in EMT in pancreatic cancer progression and suggest Embigin as a putative target for the detection and/or treatment of pancreatic cancer. © 2015 Wiley Periodicals, Inc.

Key words: Pancreatic ductal adenocarcinoma; Embigin; Epithelial to Mesenchymal Transition; transforming growth factor-β; monocarboxylate transporter2

INTRODUCTION

Embigin, a member of the immunoglobulin superfamily (IgSF), is a developmentally expressed protein. Embigin expression was first reported in F9 and PCC4 embryonal carcinoma cells [1]. During mouse embryogenesis, embigin mRNA expression is detected in the endoderm during early post implantation development, and in the gut and visceral endoderm of the somite stage [2–4]. Embigin expression is also detected during prostate gland development and tissue regression in prostate and lactating mammary gland following hormonal ablation in rat [5]. Embigin is an ancillary protein of monocarboxylate transporter 2 (MCT2). Four MCT family members (MCT1–MCT4) have been functionally characterized as $\text{H}^+$/lactate symporters mediating transport of lactic acid across the plasma membrane [6]. As such, Embigin is required to maintain the catalytic activity of MCT2 and is involved in MCT translocation to the plasma membrane [7,8]. Embigin has also been reported to be a modulator of neuromuscular junction formation [9,10]. Possible roles of Embigin include an enhancer of integrin-mediated cell to substratum adhesion, regulator of cell-extracellular matrix (ECM) interactions during development, regulator of homeostasis of normal adult tissues, and enhancer of neural cell adhesion molecule-dependent neuromuscular

Abbreviations: TGF-β, transforming growth factor-β; EMT, epithelial to mesenchymal transition; MCT2, monocarboxylate transporter2; PDAC, Pancreatic ductal adenocarcinoma.

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adhesion. Identification of a role for Embigin in cancer, however, remains elusive [4,9].

Pancreatic ductal adenocarcinoma (PDAC) is an extremely aggressive malignancy that, due to resistance to therapy, is associated with one of the worst prognoses of all human cancers [11–14]. Understanding the biology underlying the aggressive nature of pancreatic cancer may aid in solving problems, including early distant metastasis and regional invasion, frequent recurrence after radical surgery, and chemoresistance, radioresistance. The epithelial to mesenchymal transition (EMT) plays an important role in tumor invasion, and regulates metastases through various mechanisms. Expression of EMT-related proteins, such as Snail and ZEB1, is strongly correlated with cancer progression in pancreatic cancer patients [15,16].

In our studies, we have focused on exploring the role of Embigin as a regulator of cell migration and invasion and involvement in EMT in pancreatic cancer cells. We found that Embigin expression is elevated in PDAC tissues compared to normal pancreas, and silencing of Embigin reduced cell migration and invasive activity. Transforming growth factor-β (TGF-β) treatment enhanced Embigin expression, but silencing of Embigin reduced the TGF-β downstream signals, including P38, GSK3-β, and Snail/Slug, suggesting the involvement of Embigin in EMT via the TGF-β signaling pathway.

MATERIALS AND METHODS
Preparation of Human Pancreatic Tissue Specimens for cDNA Microarray Analysis, Tissue Microarray Analysis, and RT-PCR

Tissue samples were obtained from consenting individuals who underwent pancreatectomy, including the Whipple procedure or distal pancreatectomy, between January 2001 and December 2003 at Severance Hospital, Yonsei University College of Medicine. Tissue samples were examined by a pathologist and cores of cancerous and non-cancerous tissue were taken and processed for cDNA microarray analysis, tissue microarray analysis (TMA), and RT-PCR. To prepare the cDNA microarray analysis normal pancreas tissue (n = 17) and pancreatic adenocarcinoma tissue (n = 8) were examined and processed. To prepare of RT-PCR, pancreatic adenocarcinoma tissue (n = 12) and adjacent non-cancerous tissue (n = 12) were examined and processed. For histopathological diagnosis, specimens were embedded in paraffin and stained with hematoxylin and eosin (H&E) and clinical data were retrospectively reviewed. To prepare of TMA, the specimens were obtained from the paraffin blocks of PDAC (n = 33) and adjacent non-cancerous tissue (n = 20). The Ethical Committee and Institutional Review Board of Yonsei University College of Medicine approved the protocol of tissue acquisition from surgical specimens, and written informed consent was obtained from each patient.

cDNA Microarray Analysis

Total RNA from normal pancreas (n = 17) and pancreatic cancer tissue (n = 8) was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Microarray procedures were carried out according to the manufacturer’s protocols. Briefly, 6-μg aliquots of total RNA were used to prepare double-stranded cDNA. cDNAs were amplified by PCR and labeled with biotin using the IVT labeling kit (Affymetrix, Santa Clara, CA). Labeled cRNA was fragmented and hybridized to an Affymetrix GeneChip Human Genome U133 plus array. Microarrays were then washed in a Genechip Fluidics Station 450 (Affymetrix) and scanned using a Genechip Array Scanner 3000 7G (Affymetrix). Expression data were generated using Affymetrix Expression Console software version 1.1 using Mass array normalization. Expression intensity data in CEL file was normalized with the Mass array algorithm to reduce noise.

Immunohistochemistry

Immunostaining was performed using standard procedures. Sections of paraffin-embedded tissue were deparaffinized in xylene and rehydrated in a graded ethanol series. Endogenous peroxidase activity was blocked by treatment of sections with methanol containing 0.3% hydrogen peroxide at room temperature for 20 min. Antigen retrieval was performed in citrate buffer (0.01 M, pH 6.0), followed by blocking in 10% normal donkey serum for 1 h at room temperature to reduce nonspecific background staining. The tissue slides were incubated overnight at 4°C with rabbit polyclonal antibody against Embigin (Abcam, Cambridge, UK), and rabbit polyclonal antibody against MCT2 (Santa Cruz Biotechnology, Santa Cruz, CA) in antibody diluent (Gibco, Grand Island, NY). The subsequent reaction was performed using an LSAB+ Kit (Dako, Carpinteria, CA) with the manufacturer’s recommended procedure. Sections were counterstained with Mayer’s hematoxylin, dehydrated, and mounted. Between each step, there were three washing steps of 5 min each on a rocking platform in PBS. Positive expression of Embigin in acinar, ductal, and islet cells of normal and pancreatic cancer tissues was primarily in cytoplasm and cell membrane. We classified the Embigin immunoreactivity into three groups: weak expression, when Embigin positive cells were less than 20% or overall intensity is weak; moderate expression, when Embigin positive cells were less than 60% or overall intensity is moderate; and strong, when at least 90% of the cells showed positive staining of Embigin or overall intensity is strong (Supplemental Figure 1). The intensity was measured visually under a light microscopy. Three independent were visually
screened and analyzed. The mouse tissue slides were incubated overnight at 4°C with rabbit polyclonal antibody against Snail/Slug (Abcam), and mouse monoclonal antibody against Vimentin (Santa Cruz Biotechnology) in antibody diluent. The subsequent reaction was performed using an LSAB+ Kit (Dako) and sections were counterstained with Mayer’s hematoxylin, dehydrated, and mounted.

**Immunofluorescence**

Antigen retrieval was performed in the same manner as described above. Tissue slides were incubated overnight at 4°C with rabbit polyclonal antibody against Embigin (Abcam) and mouse monoclonal antibody against Keratin-19 (K-19, Santa Cruz Biotechnology) in antibody diluent. The slides were visualized using Alexa Fluor 488 goat anti-mouse IgG (Invitrogen Molecular Probes, Eugene, OR) and Alexa Fluor 555 goat anti-rabbit IgG (Invitrogen Molecular Probes) diluted (1:200) in PBS with 0.02% saponin and incubated for 20 min. Between each step, there were three washing steps of 5 min each on a rocking platform in PBS. The slides were coverslipped using an anti-fading mounting medium containing DAPI ( Vectashield H-1500; Vector Laboratories).

**Cell culture**

All human pancreatic cancer cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in the ATCC-recommended growth media and incubated at 37°C, 5% CO₂. Briefly, AsPC1 and BxPC3 cells were grown in RPMI1640 (Gibco) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and 1% antibiotics. Capan-2 cells were grown in McCoy’s 5 A medium (Gibco) with 10% FBS and 1% antibiotics. Capan-1 and CFPAC-1 cells were grown in Iscove’s Modified Dulbecco’s Medium (DMEM, Gibco) supplemented with 10% FBS and 1% antibiotics. MIA PaCa-2 cells were grown in Ham’s F12 (DMEM/F12; Gibco) with 10% FBS and 1% antibiotics, and Panc-1 cells were grown in DMEM with 10% FBS and 1% antibiotics. Human pancreatic ductal epithelial (HPDE) cells were kindly provided by Dr. Ming Sound Tsao and were grown in keratinocyte serum-free (KSF) medium with 0.2 ng/mL EGF and 30 μg/mL bovine pituitary extract (Gibco). BxPC-3 and HPAC cells were treated with TGF-β (R&D Systems) and SB-216763 (GSK-β inhibitor, Enzo Life Sciences, Farmingdale, NY) followed by incubation at 37°C, 5% CO₂.

**RT-PCR**

Total RNA from human tissues and cell pellets of harvested cultured cells were extracted using an RNeasy Mini Kit (Qiagen) following the manufacturer’s instructions. Total RNA was quantified using an ND-1000 Nanodrop spectrometer (NanoDrop Technologies, Wilmington, DE). Reverse transcription was performed using Superscript II, RNaseOUT, oligo(dT) primer, and dNTPs (all from Invitrogen, Carlsbad, CA). PCR, using Ex Taq HS DNA polymerase (TaKaRa, Shiga, Japan), was carried out for one cycle of denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 60 s; and a final extension at 72°C for 10 min. PCR products were analyzed using agarose gel electrophoresis. Primers used were human Embigin (forward) 5'-TTG TCA GTG CAA CAG GAA GC-3' and (reverse) 5'-CCA AAT AGC TCA GCA CCA CA-3' and β-actin (forward) 5'-GGC ATC TCT ACC CTG AAG TA-3' and (reverse) 5'-GGG GTG TTG AAG GTC TCA AA-3'. Quantitative RT-PCR (qRT-PCR) was performed using a TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). PCR were performed for one cycle of 94°C for 2 min and 40 cycles of 94°C for 15 s, 58°C for 20 s, and 72°C for 20 s. Relative amounts of mRNA within the samples were normalized to that of β-actin, calculated using the comparative Ct method (ΔΔCt cancer-ΔΔCt normal = ΔCt cancer-ΔCt normal), and converted to fold change. All qRT-PCR was performed in triplicate with three independent samples of total RNA. Primers used were Embigin (forward) 5'-TAC AAG TCC ACC TCT CAG AGA AG-3' and (reverse) 5'-CCC AGA TGT TGT GAA CTG GCA TG-3' and β-actin primer purchased from Applied Biosystems.

**RNA interference**

Small interfering RNA (siRNA) targeting Embigin (siGENOME SMARTpool Human EMB [133418], M-027213-00-0005) was purchased from Thermo Scientific (Dharmacon Lafayette, CO). Scramble siRNA (5'-CCU ACG CCA CCA AUU UCG U-3') and siRNA targeting GAPDH (5'-GU A UGA CAA CAG CCA CAA GTT-3') was purchased from Bioneer (Daejeon, Korea) and used as negative and positive controls, respectively, under the same conditions. Cells were transfected twice with 20 nM siRNA using Lipofectamine RNAiMAX Reagent (Invitrogen). After initial transfection, cells were re-transfected with siRNA after 72 h and gene or protein levels were determined. Cells were assayed in vitro 72 h after the second transfection.

Short hairpin RNA (shRNA) expressing plasmids targeting Embigin was constructed using the BLOCK-IT Inducible H1 RNAi Entry Vector Kit (Invitrogen), and the desired sequence was cloned into the provided plasmid, following the manufacturer’s instructions. Briefly, a single siRNA encoding the sequence (5'-GCA AAC AAA UGG GAA GUU A-3') was selected from the Embigin siGENOME SMARTpool. shRNA was designed with a top sequence of (5'-cacc gcg AAA AAA ATG GGA AGT TAT TGT TGt ctc CAA GAA TAA CT TCC AT TGT TGT C-3') and a
bottom sequence of (S'-aaa aGC AAA CAA ATG GGA 
AGT TAT TCT TGg aga CAA GAA TAA CTT CCC ATT 
TGT TTT G-3'). The shRNA was ligated into the 
pENTR/H1/TO vector and then transfected into BxPC-
3 cells. Cells expressing shRNA were selected by 
culture in Zeocin (100 µg/mL; Invitrogen). A control 
shRNA vector expressing scramble sequence was 
designed with a top sequence of (S'-cac cAT CTC 
GCT TGG GCG AGA GTA ctc CTT ACT CTC GCC 
CAA GCG AGA G-3') and a bottom sequence of (S'-aaa 
aCT CTC GCT TGG GCG AGA GTA AGg aga CTT ACT 
CTC GCC CAA GCG AGA T-3').

Western Blotting

Cells were homogenized in lysis buffer A (0.25 M 
sucrose, 20 mM Tris, pH 7.6, 1.5 mM MgCl₂, 10% 
glycerol, 1 mM EDTA, and Complete Mini Protease 
Inhibitor Cocktail (Roche Diagnostics, Indianapolis,
IN),) incubated on ice for 1 h, and centrifuged at 
12,000 rpm for 1 h at 4 °C, followed by collection of 
the supernatant. Primary antibodies to Embigin, 
Snail/Slug, and EGFR (from Abcam); MCT2, GSK3-
12,000 rpm for 1 h at 4 °C.

Inhibitor Cocktail (Roche Diagnostics, Indianapolis,
IN), followed by collection of 
the supernatant. Primary antibodies to Embigin, 
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Inhibitor Cocktail (Roche Diagnostics, Indianapolis,
IN), followed by collection of 
the supernatant. Primary antibodies to Embigin, 
Snail/Slug, and EGFR (from Abcam); MCT2, GSK3-
12,000 rpm for 1 h at 4 °C.
were conducted using protocols approved by the institutional Animal Care and Use Committee.

Statistical Analysis

Statistical analyses were analyzed using SPSS20.0 software (SPSS, Inc., Chicago, IL). The correlation between Embigin expression and cancer stage was evaluated using Spearman correlation analysis. Survival times between groups were calculated and plotted by Kaplan-Meier analysis and correlation between intensity and cancer stages were analyzed by χ² test.

RESULTS

Embigin is Overexpressed in Pancreatic Ductal Adenocarcinoma Tissues

Microarray analysis was performed with normal pancreatic tissue (NT, n = 17) and pancreatic cancer tissue (AC, n = 8). Embigin expression was elevated 3.18-fold in pancreatic cancer tissues. The mean expression level of Embigin in normal tissue was 205 (arbitrary units, P < 0.01) and 651 in cancer tissue (arbitrary units, P < 0.01) (Figure 1a). We isolated total RNA from cancer and normal tissues and compared relative Embigin mRNA expression by RT-PCR. Ten of twelve patients showed elevated Embigin expression in cancer tissue (Figure 1b).

We next carried out immunohistochemical analysis of paraffin sections of pancreatic cancer and normal tissues (Figure 2). Normal tissues were composed of acini, islet cells, and ducts without cancerous or metaplastic changes. Embigin was moderately stained in islet cells and duct cells, and weakly stained in acini (Figure 2a). Of 20 samples of normal tissue, Embigin was weakly expressed in 19 (95%) and moderately expressed in one (5%). In cancer tissue, Embigin expression was moderate to strong in cancer cells and preneoplastic lesions such as metaplastic and dysplastic ducts (Figure 2b). Of 33 pancreatic cancer specimens, Embigin expression was moderate or strong in 20 (60.6%) and 10 (30.3%) samples, respectively, and weak in 3 (9.1%) cases. Significant associations between Embigin expression intensity and clinicopathological parameters were assessed and we found no significant association between intensity and survival time (P = 0.112) or intensity and cancer stage (P = 0.542).

As Embigin is required for translocation of MCT2 to the plasma membrane, we analyzed the expression of
MCT2 in pancreatic cells. Immunostaining showed MCT2 expression in the acini but not in the islet cells of normal pancreas. Meanwhile, MCT2 expression was strong in cancer cells and preneoplastic lesions where Embigin was strongly expressed (Figure 2c, d). No significant association between intensity and survival time ($P = 0.124$) or intensity and cancer stage ($P = 0.017$) were found in relation to MCT2 expression. Survival curves were plotted by Kaplan–Meier analysis (Supplement Figure 2).

We performed immunofluorescence with Embigin and a ductal cell marker, K-19, to visualize localization. In normal tissue, Embigin was moderately visualized in islet cells and duct cells and weakly visualized in cells of the acini; K-19 was strongly visualized in duct cells (Figure 2e). In
cancer tissue, Embigin expression was visualized in cancer cells in which K-19 was also expressed (Figure 2f).

Silencing of Embigin Reduces Cell Migration and Invasive Ability

Total RNA was isolated from the pancreatic cancer cell lines AsPC1, BxPC-3, Capan-1, Capan-2, CFPEC-1, HPAC, MIA PaCa-2, and Panc-1 and from the normal pancreatic cell line HPDE. Embigin expression level was analyzed by qRT-PCR. Embigin was expressed in all pancreatic cancer cell lines and, at a lower level, in the normal cell line (Figure 3a).

To examine the functional role of Embigin, we silenced Embigin expression in the pancreatic cancer cell line BxPC3 and HPAC using siRNA or shRNA. The remaining Embigin mRNA in BxPC-3 and HPAC was analyzed by qRT-PCR and was 12.9% ± 1.29 (P < 0.01) and 12.3% ± 2.3 (P < 0.01) respectively (Figure 3b). The remaining Embigin mRNA in BxPC-3 transfected with shRNA targeting Embigin was analyzed by qRT-PCR and was 26.1% ± 4.11 (P < 0.01) (Figure 3c).

Overall, cells treated with siRNA targeting Embigin became more rounded, and cells with tapered ends disappeared (Figure 3d). Embigin silencing led to a decrease in BxPC-3 cell proliferation of 27.2% (P < 0.01) (Figure 3e). Silencing led to a decrease in cell migration in BxPC-3 and HPAC of 65 ± 13% and 32 ± 9.8% (P < 0.05) and in cell invasion of 32 ± 9.8% and 44 ± 17.3% (P < 0.05) respectively (Figure 3f). The activity of MMPs is critical for cancer cells to invade through extracellular matrices. We performed zymography to assay the activities of MMP-2 and MMP-9 and observed that secretion of both active MMPs was reduced in BxPC-3 and HPAC cells transfected with Embigin siRNA (Figure 3f). When we tested wound-healing ability of the BxPC-3 cells, wounds were healed in 21 h by control, scramble siRNA-transfected cells, whereas the wound-healing process required 28 h in Embigin siRNA-transfected cells (Figure 3h).

Embigin Involvement in EMT via TGF-β Signaling

To examine downstream signaling, we transfected BxPC-3 and HPAC cells with siRNA targeting Embigin and screened differentially expressed genes. We noticed that EMT-related genes PI3K, GSK3β, and Snail/Slug were reduced in expression due to Embigin silencing (Figure 4a). To determine whether Embigin is controlled by TGF-β, BxPC-3 cells were treated with increasing concentrations of TGF-β1. Elevated expression of Embigin was observed at a concentration of 1.25 and 2.5 ng/mL TGF-β (Figure 4b). Reduced expression was observed at concentrations higher than 5 ng/mL TGF-β which might induce apoptosis, followed by reduction in Embigin expression. We treated BxPC-control shRNA cells and BxPC-Embigin shRNA cells with 2.5 ng/mL TGF-β for 0, 1, 2, and 4 d. TGF-β induced elevated expression of Embigin together with Snail/Slug and Vimentin. In contrast, the absence of Embigin led to decrease in Snail/Slug and Vimentin expression (Figure 4c). Cells treated with SB-216763, GSK-3β inhibitor for 0, 2, 4, 8, 16, and 32 nM decreased not only GSK-3β but also Embigin and Snail/Slug, dose dependently (Figure 4d).

Silencing of Embigin Increased Intracellular L-Lactate Concentration and Reduced MCT2 Expression in Plasma Membrane

To examine the possible relationship between Embigin expression and lactate transport, the concentration of intracellular L-lactate was measured in Embigin siRNA-transfected BxPC-3 and HPAC cells with or without TGF-β (2.5 ng/mL). Silencing Embigin markedly increased intracellular L-lactate concentration by 1.50 ± 0.02-fold (P < 0.01) and 1.44 ± 0.03-fold (P < 0.01) respectively, in Embigin siRNA-transfected than in scramble siRNA-transfected cells. Treatment of TGF-β decreased intracellular L-lactated concentration by 0.78 ± 0.03-fold (P < 0.05) and 0.81 ± 0.04-fold (P < 0.05) in scramble siRNA-transfected cells whereas the treatment of Embigin siRNA transfected cells merely decreased from 1.50 ± 0.02 and 1.44 ± 0.03-fold to 1.42 ± 0.12-fold (P < 0.05) and 1.39 ± 0.06-fold (P < 0.05) respectively (Figure 5a). We then examined whether Embigin silencing influenced the level of MCT2 in cells and slight decrease in the level of MCT2 was seen in Embigin siRNA-treated BxPC-3 and HPAC cells (Figure 5b). Subcellular fractionation was performed and in cells transfected with Embigin siRNA, MCT2 level in plasma membrane was slightly decreased compared to that of scramble siRNA transfected cells (Figure 5c). Thus, Embigin is involved in L-lactate secretion, and silencing Embigin reduced MCT2 expression level in plasma membrane.

Silencing of Embigin Inhibited Tumor Growth In Vivo

BxPC-control shRNA cells and BxPC-Embigin shRNA cells (5 × 106 cells/site) were injected subcutaneously into two groups of mice. Tumor size was measured every 7 d (volume = length × width2/2). Mice were sacrificed at the end of the experiment, and tumors were collected for microscopic analysis. The average volume of tumors in mice injected with BxPC-control cells was 641.9 ± 106.8 mm3 (P < 0.01) and the average volume in the group injected with BxPC-Embigin shRNA was 423.1 ± 50.6 mm3 (P < 0.01) (Figure 6b,c). Tissues were stained with hematoxylin and eosin (H&E), and cell morphology was observed under a microscope and we found no difference in morphology (Figure 6d). We immunostained Embigin, MCT2, Snail/Slug, and Vimentin on serial sections of mouse tissues and the BxPC-Embigin
Figure 3. Continued.
Figure 3. Silencing of Embigin changes cell morphology and reduces proliferation, migration, invasion, wound-healing activity, and MMP secretion. (a) Total RNA from the indicated human pancreatic cancer cell lines and the normal pancreatic cell line HPDE was isolated, and Embigin mRNA expression was examined by qRT-PCR. (b) The siRNA efficiency was analyzed by qRT-PCR and the remaining Embigin mRNA in BxPC-3 and HPAC cells was 12.9% ± 1.23% (**, P < 0.01) and 12.3% ± 2.3% (**, P < 0.01), respectively. (c) The remaining Embigin mRNA in BxPC-3 cells transfected with shRNA targeting Embigin was analyzed and was 26.1% ± 4.11% (**, P < 0.01). (d) Suppression of Embigin expression by siRNA (20 nM) changed the cell morphology from tapered ends (arrowheads in left panel, scrambled siRNA) to rounded ends (right, Embigin siRNA). (e) Cell proliferation was decreased by 27.2% ± 3.9% (**, P < 0.01) in BxPC-3 Embigin shRNA-transfected cells. (f) Migration and invasion assays were carried out on scramble siRNA- and Embigin siRNA-transfected cells to analyze cell motility and secretion of active MMPs. Silencing led to a decrease in cell migration in BxPC-3 and HPAC cells of 65% ± 13% and 32% ± 9.8% (*, P < 0.05) and in cell invasion of 32% ± 9.8% and 44% ± 17.3% (*, P < 0.05) respectively. (g) Reduced expression of active MMP-2, and -9 was observed in Embigin siRNA-transfected cells by zymography. (h) Cultured cells were scratched with a yellow tip, after which control cells repaired the wounds in 24 h, while BxPC-3 siRNA-transfected cells did so in 7 h.

Molecular Carcinogenesis
Figure 5. Embigin silencing reduces L-lactate secretion (a) Intracellular L-lactate concentration was measured in BxPC-3 and HPAC cell lines. The concentration was 1.50 ± 0.02-fold (**, P < 0.01) and 1.44 ± 0.03-fold (**, P < 0.01) greater, respectively, in Embigin siRNA-transfected than in scramble siRNA-transfected cells. Treatment TGF-β decreased intracellular L-lactate concentration by 0.78 ± 0.03-fold (*, P < 0.05) and 0.81 ± 0.04-fold (*, P < 0.05) in scramble siRNA-transfected cells whereas the treatment of Embigin siRNA transfected cells merely decreased from 1.50 ± 0.02 and 1.44 ± 0.03-fold to 1.42 ± 0.12-fold (*, P < 0.05) and 1.39 ± 0.06-fold (*, P < 0.05) respectively. (b) MCT2 levels in Embigin siRNA-transfected cells were slightly lower than in scramble siRNA-transfected cells. (c) MCT2 levels in Embigin siRNA or scramble transfected cells subcellular fractions. F1, cytosol; F2, membrane.
shRNA group showed decrease in MCT expression in plasma membrane as well as decrease in Snail/Slug, and Vimentin was observed (Figure 6e).

**DISCUSSION**

Embigin expression has been observed during early mouse embryogenesis and rat neuromuscular junction formation. Detectable amounts of Embigin are expressed in adult rat tissues, including heart, liver, lung, and brain. Embigin has also been detected in human prostate and mammary cancer cell lines and mouse embryonal carcinoma cells [5]. Embigin may be involved in integrin-mediated cell-substratum adhesion, as well as in neural cell sprouting during neuromuscular junction formation, yet expression of Embigin in other organs or disease model has not been investigated. Basigin, another member of the IgSF, plays a role in lymphocyte responsiveness, neurological functions in early development, expression of MCT, and regulation of MMPs [17–21].

Investigation of the functional role of Basigin in cancer has focused on the findings that Basigin induces MMPs and promotes angiogenesis, activates PI3K/Akt, enhances cell proliferation, and interacts with MCT to deregulate lactate production [22–25].

In this study, we found that Embigin is strongly expressed in human PDAC and regulates cell motility,
MMP secretion, and TGF-β downstream signals. The presence of Embigin in adult human pancreas or pancreatic cancer tissue has not been previously reported. In this study we first analyzed human tissue cDNA microarrays and showed that Embigin expression was significantly elevated in the cancer tissue. Determination of Embigin expression in normal and cancerous pancreatic tissue of 12 patients by RT-PCR, showed that Embigin expression was relatively high in cancer tissues, in agreement with the microarray analysis. The immunohistochemical analysis with normal specimens supports the evidence that Embigin is expressed weakly in normal pancreas acinar cells and merely in normal ductal cells and is not a ductal cell specific protein.

In this study, we found that silencing Embigin significantly reduced cell migration and invasion. Decreases in MMP-2 and MMP-9 may further reduce invasiveness, which implies that Embigin is functionally involved in cell motility. Because Embigin is involved in cell to substratum adhesion, and our data show that Embigin regulates cell motility, we focused on the possible relationship between Embigin and EMT [4]. EMT occurs during embryonic development, as well as in cancer progression. During EMT, epithelial cells lose cell-cell adhesion and polarity, and acquire cell migratory and invasive properties [26]. In pancreatic cancer, EMT markers, such as N-cadherin, Vimentin, and transcription factors, including Snail and Slug, have shown increased expression compared with normal pancreas [15,27,28]. It was previously reported that the IgSF member Basigin promotes EMT via TGF-β signaling [3,29,30]. Silencing Embigin reduced the TGF-β signal cascade of GSK3-β, PI3K, and Snail/Slug. The TGF-β family is known to include EMT inducers, and TGF-β1 has been shown to induce EMT via the Snail/Slug transcription factors [31,32]. Treatment of BxPC-3 cells with TGF-β markedly increased Embigin levels at a concentration of 2.5 ng/mL, indicating that TGF-β is an upstream regulator of Embigin expression.

Treatment of cell with TGF-β and GSK3-β inhibitor modulate not only Embigin expression but also other EMT markers as Snail/Slug and Vimentin expression. Altering gene expression by shRNA suggested that in the presence of Embigin, TGF-β increases Embigin and Snail/Slug expression. In the absence of Embigin, TGF-β increases Snail/Slug expression but the expression level is lower than that of Embigin expressing cells. Vimentin was used as a EMT marker and the expression level was decreased by the suppression of Embigin expression.

In long-term in vivo silencing, tumor volumes were smaller in Embigin-silenced groups than in control vector groups. For long-term silencing, we adopted a shRNA system, and silencing of Embigin inhibited cell proliferation both in vitro and in vivo. Even though the shRNA silencing yield was less than that of siRNA, Embigin shRNA inhibited cell proliferation by approximately 35% in vitro. It is not clear how silencing Embigin changed cell proliferation rates and tumor volume in vivo. Embigin is an ancillary protein of MCT2, and is involved in translocation of monocarboxylate. We immunostained Embigin and MCT2 on serial sections of mouse tissues and noted decreases in MCT expression in plasma membranes. Cancer cells produce excess lactate and pyruvate for propagation (the Warburg Effect), and silencing of Embigin, followed by a decrease in L-lactate transport in the long-term, may explain the inhibition in cell proliferation and mouse tumor propagation [9,33–35]. We immunostained Snail/Slug, and Vimentin on serial sections of mouse tissues and observed decreases in Snail/Slug, and Vimentin. However, metastasis was not observed in either group.

CONCLUSION

Our study reports for the first time that Embigin expression is elevated in PDAC, based on cDNA microarray and tissue mRNA analyses, as well as assays of protein level. Further, novel functions of Embigin in regulation of cell proliferation, migration, invasion, wound healing, MMPs, TGF-β downstream transcription factors, and L-lactate uptake were demonstrated, as well as the first demonstration of co-expression of Embigin and MCT2 in pancreatic cancer tissues. We propose that therapeutic targeting of Embigin may contribute to repression of pancreatic cancer progression.

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REFERENCES


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