Neurotrophic Factor Artemin Promotes Invasiveness and Neurotrophic Function of Pancreatic Adenocarcinoma In Vivo and In Vitro

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Objectives: The aim of this study was to investigate the effect of the neurotrophic factor Artemin on neuroplasticity and perineural invasion of pancreatic adenocarcinoma.

Methods: Artemin expressions were detected in human pancreatic adenocarcinoma tissues by Western blot and immunohistochemistry. Artemin overexpression and RNA interference in the pancreatic cancer cell lines were performed to evaluate the effects of Artemin on cell proliferation, invasion, and neurotrophic activity in vitro and in nude orthotopic transplantation tumor models.

Results: Artemin expression in pancreatic cancer tissues was related to the incidence of lymphatic metastasis and perineural invasion as well as the mean density and total area of nerve fibers. Overexpression of Artemin in pancreatic cancer cell lines improved colony formation, cell migration, matrigel invasion, and neurotrophic activity in vitro. This overexpression also increased the volume of nude orthotopic transplantation tumors; promoted cancer cell invasion of the peripheral organs, nerves, vessels, and lymph nodes; and stimulated the proliferation of peritumoral nerve fibers. Artemin depletion by RNA interference had an inhibitory effect mentioned previously.

Conclusions: Artemin could promote invasiveness and neurotrophic function of pancreatic adenocarcinoma in vivo and in vitro. Therefore, Artemin could be used as a new therapeutic target of pancreatic carcinoma.

Key Words: pancreatic carcinoma, neurotrophic factor, Artemin, perineural invasion, RNA interference

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Pancreatic adenocarcinoma is one of the most aggressive malignant tumors with a very poor prognosis. The overall 5-year survival rate can be less than 5%. Perineural invasion (PNI) is a major characteristic of pancreatic adenocarcinoma. Studies have shown a high incidence of PNI (approximately 51%–100%) in pancreatic adenocarcinoma[1]; PNI is present in a small primary lesion. Although margins do not contribute to tumor growth, tumor cells possibly spread along the nerves. Another study[2] has shown that PNI in pancreatic adenocarcinoma can cause intractable pain and lead to recurrence and metastasis of cancers; hence, PNI is considered as an independent prognostic factor. However, the molecular mechanism of PNI in pancreatic adenocarcinoma

remains unclear, and no therapy has been developed to target this process.

Artemin is a neurotrophic factor discovered in 1998 and belongs to the glial cell line–derived neurotrophic factor (GDNF) family.[3] Artemin enhances the survival of several types of neurons and is important in the development and differentiation of central and peripheral nervous systems.[4] Artemin also functions as a guidance molecule and induces migration and axonal projection from sympathetic neurons.[5] Furthermore, Artemin reduces neuropathic pain and is involved in restoring neural damage.[6] Abnormal expressions of Artemin in tumors, such as pancreatic carcinoma, endometrial cancer,[7] breast cancer,[8] and non–small-cell lung carcinoma,[9] can affect tumorigenesis, invasiveness, and drug resistance to certain chemotherapies. However, the function of Artemin in PNI lacks a comprehensive study. No evidence based on in vivo experiments has been found to illustrate the function of Artemin in pancreatic adenocarcinoma. In this study, the functions of Artemin were investigated in pancreatic cancer tissues and cell lines. This study is the first to identify these functions in nude orthotopic transplantation models. The expression of Artemin was also inhibited by RNA interference (RNAi) to determine the potential therapeutic value in pancreatic adenocarcinoma.

MATERIALS AND METHODS

Tissues, Animals, and Cell Lines

Eleven pairs of tumor and peritumoral tissues were collected from the surgical specimens of pancreatic ductal adenocarcinoma in Changhai Hospital, Shanghai, China, in October 2008 and stored in a refrigerator at −80°C for the subsequent Western blot analysis. A total of 100 samples of paraffin-embedded pancreatic cancer tissues and 23 peritumoral tissues from surgical specimens were selected for immunochemistry. The collected specimens were obtained from 2005 to 2009 in Changhai Hospital, Shanghai. The specimens were obtained from patients aged 29 to 82 years (66 men and 34 women). A total of 60 classic ductal adenocarcinoma and 40 adenosquamous carcinoma specimens were collected. This study was approved by the ethics committee of the Second Military Medical University. Before the experiments were conducted, the subjects were informed of the objectives, requirements, and procedures of the experiments. All of the subjects provided a written informed consent to participate in the study.

A total of 24 BALB/C-nu/nu mice (12 males and 12 females aged 4–6 weeks and weighed 20–26 g) were provided by the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences and bred in a specified pathogen-free condition with constant humidity (45%–50%) and temperature (25°C–28°C). These mice were divided into 4 groups with 6 mice each. This study was performed in strict compliance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China. The protocol was approved by the ethics committee of

the Second Military Medical University. All of the surgical operations were performed under sodium pentobarbital anesthesia; efforts were undertaken to minimize animal suffering.

Human pancreatic cancer cell lines (MIA-PaCa-2, BxPC3, PANC-1, Capan-1, SW1990, AsPC-1) were obtained from Institute of Cellular Research, Chinese Academy of Science, Shanghai, China, and routinely grown in recommended growth media, supplemented with 10% of fetal bovine serum, 100 U/mL of penicillin, and 100 ig/mL of streptomycin at 37°C and saturated with 5% of CO_2 in a humid atmosphere. The PC12, a cell line derived from a pheochromocytoma of the rat adrenal medulla, was routinely maintained in Dulbecco Modified Eagle Medium containing 10% of equine serum and 5% of fetal bovine serum at 37°C and saturated with 5% of CO_2 in a humid atmosphere.

Reagents

Artemin-goat polyclonal antibody (R&D Systems, Minneapolis, MN); growth-associated protein 43 (GAP-43)-rabbit polyclonal antibody (Millipore, Bedford, MA); glyceraldehyde-3-phosphate dehydrogenase-mouse monoclonal antibody (EarthOx, San Francisco, CA); antigoat, antimouse, antirabbit immunoglobulin G horseradish peroxidase-linked antibodies (MingRui, Shanghai, China); electrochemiluminescence immunoblot detection reagents (Tiangen, Beijing, China); RPMI-1640 medium and Dulbecco Modified Eagle Medium (Solarbio, Beijing, China); fetal bovine serum; equine serum; trypsin + ethylene diamine tetraacetic acid; G418 (Gibco, Grand Island, NY); Lipofectamine 2000 (Invitrogen, Carlsbad, CA); Blasticidin-S (Merck, Darmstadt, Germany); and methylthiazolyldiphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich, MO) were prepared for experimental requirements.

Western Blot

The total proteins of tumor tissues, peritumoral tissues, and in vitro–passaged human pancreatic cancer cell lines were extracted according to the recommended protocol. Artemin antibody was used at a dilution of 1:2000 (0.1 µg/mL), and glyceraldehyde-3-phosphate dehydrogenase antibody was used at a dilution of 1:3000 at 4°C overnight. Antibody was detected via an enhanced chemiluminescence reaction. Film scanning was conducted using an automatic gel imaging instrument (Bio-Rad, CA), and densitometric analysis was performed using the Image J software (NIH Image, Bethesda, MD).

Immunohistochemistry

A total of 100 samples of pancreatic adenocarcinoma and 23 samples of peritumoral tissues were selected for tissue microarrays. Artemin antibody was used at a working dilution of 1:20 at 4°C overnight to detect Artemin expression by immunohistochemistry (IHC). The paraffin sections of the pancreatic adenocarcinoma samples were detected for GAP-43. The GAP-43 antibody was diluted to 1:1000 at 4°C overnight. Normal goat serum was used as a surrogate control of the primary antibody; phosphate buffered saline (PBS) was used as a blank control. The secondary antibody was diluted to 1:200 at room temperature for 30 minutes; 3,3′-diaminobenzidine reaction and microscopic analysis were then conducted. A Motic microscopic imaging system (Motic, Xiamen, China) was used to collect IHC images, and Image J software was used to measure the maximum area of nerve fibers per section. The same software was used to count the number of nerve fibers that were positive for GAP-43 and the number of nerve fibers per square centimeter.

Construction, Transfection, and Selection of Artemin Eukaryotic Overexpression and MicroRNA Plasmids

Artemin eukaryotic overexpression plasmid pCDNA3.1-Artemin was constructed based on the Artemin gene sequence in GenBank and transfected into PANC-1 and BxPC3 cell lines. Four pairs of microRNA (miRNA) oligos targeting Artemin sequences (Table 1) were designed to construct pCDNA6.2-Artemin-miRNA and transfected into PANC-1 cell. The BxPC3 cell was not transfected with Artemin-miRNA plasmid because of its low Artemin expression. Cells were transfected with Lipofectamine 2000 according to the recommended protocol. An empty vector pCDNA3.1 and pCDNA6.2 were used as controls. After transfection, antibiotics were used for 14 days to select colonies and establish stable lines. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blot were then performed to detect the expression levels of Artemin in selected monoclonal stably transfected cell lines.

Cellular Biological Function Test

MTT Growth Assay

In brief, 1.5×10^3 cells/well were seeded in 96-well plates; 8 replicate wells were provided for each group. After 24, 48, 72, 96, 120, and 144 hours of incubation, MTT was added (10 µL/well) for 4 hours. Formazan products were solubilized with acidic isopropanol, and the optical density was measured at 570 nm. Optical density values at different incubation periods were compared with those at 0 hour of incubation to determine the proliferation rate curve.

Flow Cytometry

The cells were seeded at a density of 1×10^6 cells/well in 6-well plates. At 48 hours after seeding, the confluence rate of cells reached 80%. Different groups of cells were collected and fixed in 70% of ethanol at 4°C overnight. Approximately 100 µL of 0.2 mg/mL of ribonuclease/PBS was added at 37°C for 1 hour. Approximately 0.5 mL of 1 mg/mL of propidium iodide (PI)/PBS was added at room temperature for 15 minutes. Flow cytometry was performed to detect the cell cycles, and the Light cycle software was used for analysis. The cells in the exponential phase

TABLE 1. miRNA Oligo Sequences of the Artemin Gene

miR-1	5′-TGCTGTGGAGAGGCCTCCAAGTCCAA**GTTTTGGCCACTGACTGAC**TTGGACTTAGGCCTCTCCA -3′
miR-1	5′-CCTGTGGAGAGGCCTAAGTCCAA**GTCAGTCAGTGGCCAAAAC**TTGGACTTGGAGGCCTCTCCAC -3′
miR-2	5′-TGCTGTGAAGGAGACCGCTTCGTAGC**GTTTTGGCCACTGACTGAC**GCTACGAAGGTCTCCTTCA -3′
miR-2	5′-CCTGTGAAGGAGACCTTCGTAGC**GTCAGTCAGTGGCCAAAAC**GCTACGAAGCGGTCTCCTTCAC -3′
miR-3	5′-TGCTGTTGACGTCCATGAAGGAGACC**GTTTTGGCCACTGACTGAC**GGTCTCCTATGGACGTCAA -3′
miR-3	5′-CCTGTTGACGTCCATAGGAGACC**GTCAGTCAGTGGCCAAAAC**GGTCTCCTTCATGGACGTCAAC -3′
miR-4	5′-TGCTGTGTTGACGTCCATGAAGGAGA**GTTTTGGCCACTGACTGAC**TCTCCTTCGGACGTCAACA -3′
miR-4	5′-CCTGTGTTGACGTCCGAAGGAGA**GTCAGTCAGTGGCCAAAAC**TCTCCTTCATGGACGTCAACAC -3′

were collected, and the cell density was adjusted to 1×10^6 cells/mL by using a combined buffer. Approximately 5 μL of Annexin V/APC and 10 μL of 20 μg/mL of PI were added to 100 μL of cell suspension. The control system contained only Annexin V/APC or PI. The mixture was then placed at room temperature in the dark for 15 minutes. The PBS (400 μL) was added; apoptosis was determined by flow cytometry.

Colony-Forming Test

The cells in each group were seeded at 1×10^3 cells/well in a 6-well plate in triplicate. After 10 days, the cells were stained, and the number of colonies was counted. The colony-forming rate was calculated using the following equation: colony-forming rate = number of colonies/number of seeded cells $\times$ 100%.

Scratch Test

The cells (1×10^5 cells/well) were seeded in 6-well plates and incubated for 24 hours (3 wells for each group). A line was drawn in the middle of the confluent cell monolayer in the well. This procedure was conducted in triplicate. A serum-free culture medium was then added. Cell migration was determined after 24 hours.

Matrigel-Based Invasion Assay

Invasion assay was performed using Matrigel invasion chambers (BD Bio, Germany) and used according to the manufacturer's instructions. In brief, the Matrigel was hydrated with serum-free RPMI-1640 and incubated. The cells (5×10^4 cells) were seeded in the upper chamber of the invasion chambers and incubated for 72 hours at 37°C in 5% of CO_2 atmosphere. The noninvading cells were removed from the upper surface of the membrane by wiping with a cotton-tipped swab. The cells that adhered to the lower surface were fixed in methanol and stained with crystal violet. The 5 high-power fields of the invading cells were randomly counted microscopically to calculate the number of invading cells per high-power field.

Induced Differentiation Test of PC12 Cells

Polylysine was pre-embedded in 6-well culture plates. Undifferentiated PC12 cells were planted at a concentration of 1×10^3 cells/well. The PC12 is a cell line derived from a pheochromocytoma of the rat adrenal medulla. The PC12 cells stop dividing and undergo terminal differentiation when treated with a nerve growth factor; hence, the PC12 cell line is a useful model of nerve cell differentiation. The Artemin overexpression and Artemin miRNA PANC-1 cells were cultured in complete culture media for 24 hours after the cells entered the exponential phase. Supernatants were collected and added to 6-well plates containing PC12 cells, with untreated cells as a control group. Culture media were changed at an interval of 3 days. The images of the cultures were obtained after 10 days.

Orthotopic Transplantation Nude Models

A total of 24 BALB/c-nu mice were divided into 4 groups (transplanted Artemin-overexpression PANC-1 cell, pCDNA3.1 control PANC-1 cell, Artemin miRNA PANC-1 cell, and pCDNA6.2 control PANC-1 cell, respectively). The mice were anesthetized and dissected to expose the pancreas and spleen. Approximately 50 μL of cell suspensions collected from the 4 groups (5×10^7 cells/mL) were injected subcapsularly in the tail of the pancreas. After 8 weeks, the mice were killed for the next detection. Pathological characters including invasion and metastasis were determined by 2 experienced pathologists independently.

Serial sections of the internal organs of mice were made and observed by them under microscope.

Statistical Analysis

Statistical analyses were performed using statistical product and service solutions 12.0 software. Continuity data and noncontinuity data were analyzed using t test and χ^2 test, respectively.

RESULTS

Artemin Protein Expression in Pancreatic Cancer Tissues and Pancreatic Cancer Cell Lines

Western blot results showed that Artemin protein was expressed in pancreatic and peritumoral tissues (Fig. 1A); the expression in tumor tissues was significantly higher than that in peritumoral tissues ($P < 0.05$). Western blot analysis showed varying expressions of Artemin in the pancreatic cancer cell lines MIA-PaCa-2, BxPC3, PANC-1, Capan-1, SW1990 and AsPC-1 (Fig. 1B). A higher expression was found in cell lines with high invasive potential such as SW1990 and AsPC-1 compared with that in cell lines with low invasive potential such as BxPC3.

The IHC showed that Artemin was localized in the cytoplasm (Fig. 2). In tumor tissues, some parts of the tubular complex, insular cells, and nerve tissues were positively expressed. The positive proportion of Artemin in tumor tissues (71.0%, 71/100) was higher than that in peritumoral tissues (43.5%, 10/23;
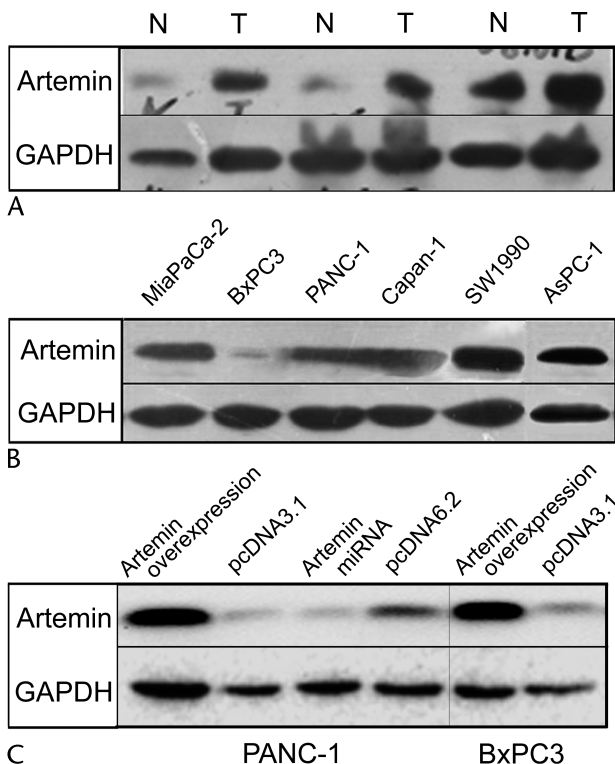

FIGURE 1. Western blot analysis of Artemin in pancreatic cancer tissues and pancreatic cancer cell lines. A, Artemin expression in tumor tissues (T) was higher than that in peritumoral tissues (N). B, Various expressions of Artemin in 6 kinds of pancreatic cancer cell lines (high expression in SW1990 and AsPC1, low expression in BxPC3). C, Artemin expression in PANC-1 and BxPC3 cell lines after being transfected with Artemin-overexpression plasmid, Artemin miRNA plasmid, and their respective empty vector plasmids.
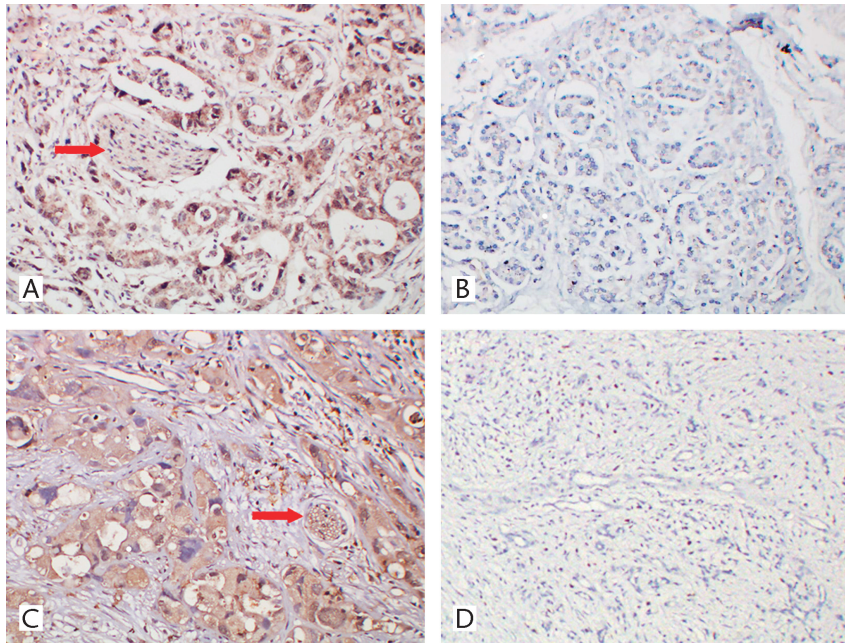
FIGURE 2. Determination of Artemin expression by IHC. A, Artemin expressed in the cytoplasm of pancreatic ductal adenocarcinoma and the nerve fibers in the stroma showed positive expression (→; IHC, 100×). B, Peritumoral tissue of pancreatic ductual adenocarcinoma showed negative expression of Artemin (IHC, 100×). C, Artemin expressed in the cytoplasm of pancreatic adenosquamous carcinoma and the nerve fibers in the stroma showed positive expression (→; IHC, 100×). D, Peritumoral tissue of pancreatic adenosquamous carcinoma showed negative expression of Artemin (IHC, 100×).

$P < 0.05$); this expression was associated with the incidence of lymphatic metastasis and PNI but was not associated with sex, age, histological types (ductal adenocarcinoma or adenosquamous carcinoma), and peripheral organ invasion (Table 2).

Significant hypertrophy and hyperplasia of nerve fibers were observed in the stroma of pancreatic tumor and peritumoral tissues. The GAP-43 was positively expressed in the nerve sheath. The expression of Artemin was associated with the mean nerve

TABLE 2. Characteristics of Patients and Tumors According to Artemin Expression

| Characteristics		n	Artemin		P
			Positive	**Negative**	
Sex	Male	66	47	19	0.948
	Female	34	24	10	
Age, years	<50	16	11	5	0.856
	50–70	67	47	20	
	>70	17	13	4	
Histologic variant	Ductal adenocarcinoma	60	44	16	0.529
	Adenosquamous carcinoma	40	27	13	
Tumor location of pancreas	Head and neck	58	42	16	0.714
	Tail	42	29	13	
Tumor size, cm	≤3	29	23	6	0.401
	3–6	44	31	13	
	≥6	27	17	10	
Histologic grade	Well/moderately differentiated	80	55	25	0.321
	Poorly/undifferentiated	20	16	4	
Lymph node metastasis	Positive	52	41	11	0.036*
	Negative	44	26	18	
Surrounding organs' invasion	Positive	53	38	15	0.870
	Negative	47	33	14	
PNI	Positive	68	56	12	0.000*
	Negative	32	15	17	
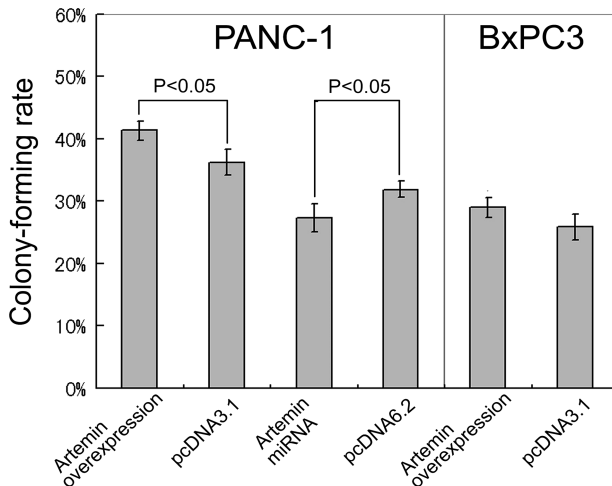
*$P < 0.05$.

FIGURE 3. Effect of Artemin on colony-forming rate of pancreatic cancer cells. The colony-forming rate of Artemin overexpressing PANC-1 cells was significantly higher than that in the control group ($P < 0.05$). In contrast, Artemin-depleted PANC-1 cells showed a lower colony-forming rate than that in the control group ($P < 0.05$). There was no significant difference in the colony-forming rate between Artemin overexpressing and control BxPC-3 cells ($P > 0.05$). Results are expressed as mean (SD).

fiber density and the maximal area of nerve fiber, which were measured by Image J software (Table 2).

Determination of Efficiency of Overexpression and RNAi by qRT-PCR and Western Blot

The monoclonal cell lines with the highest overexpression and RNAi efficiencies were selected by qRT-PCR and Western blot (Fig. 1C). The qRT-PCR results showed that the Artemin concentration increased 3 times in the overexpressed cell line compared with that in the control group. The miRNA-1 and miRNA-2 efficiently inhibited Artemin expression, which could be lowered to 20.89% when detected by qRT-PCR, at an mRNA inhibition rate of 79.11%.

Cellular Biological Function Test

The MTT assay was used to determine the cell growth curve. No difference was found in the transfected groups (data not shown). Flow cytometry was conducted to trace the proportions of the cells in the G1, S, and G2 phases. No difference was found among the groups. Moreover, no difference was found among the proportions of live cells and in the early apoptotic, late apoptotic, and necrotic cells (data not shown).

Colony-forming test results showed that the colony-forming rate of PANC-1 cells had significant difference between Artemin overexpression group, Artemin RNAi group, and their respective control groups ($P < 0.05$). There was no significant difference in the colony-forming rate between Artemin overexpression and control BxPC3 cell ($P > 0.05$) (Fig. 3).

Cell migration test results revealed that the cells in all groups reached a confluent growth at 24 hours after these cells were seeded in 6-well plates. At 24 hours after the miRNA-transfected PANC-1 and BxPC3 cells were scratched, migration was slightly observed compared with that in the 2 empty vector groups; the migrating distance was 20% to 30% of scratching width. By contrast, a high number of cells transfected with Artemin migrated beyond the scratching line and achieved a confluent rate of greater than 60% to 100% (Fig. 4).

Matrigel-based invasion assay was performed to determine the effect of Artemin on pancreatic carcinoma invasion. The induced expression of Artemin significantly promoted pancreatic
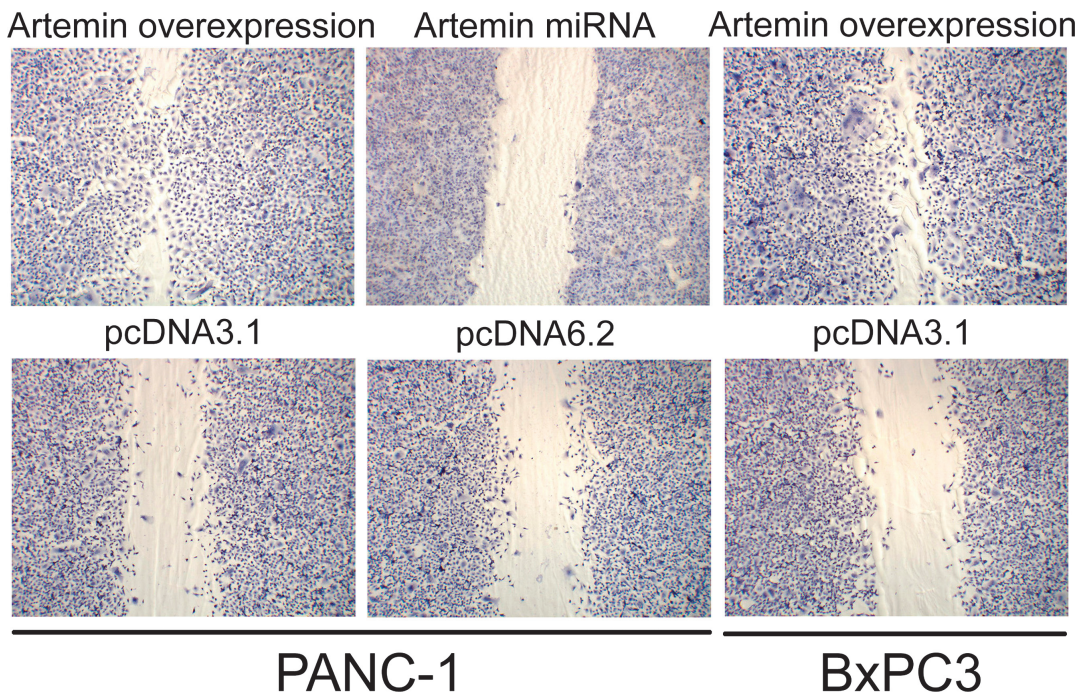

FIGURE 4. Effect of Artemin on the migration of pancreatic cancer cells. Twenty-four hours after scratching, the Artemin-depleted PANC-1 cells showed inconspicuous migration tendency compared with the empty vector control. Contrastingly, a big proportion of Artemin overexpressing PANC-1 and BxPC3 cells migrated much more than those in their respective control groups.
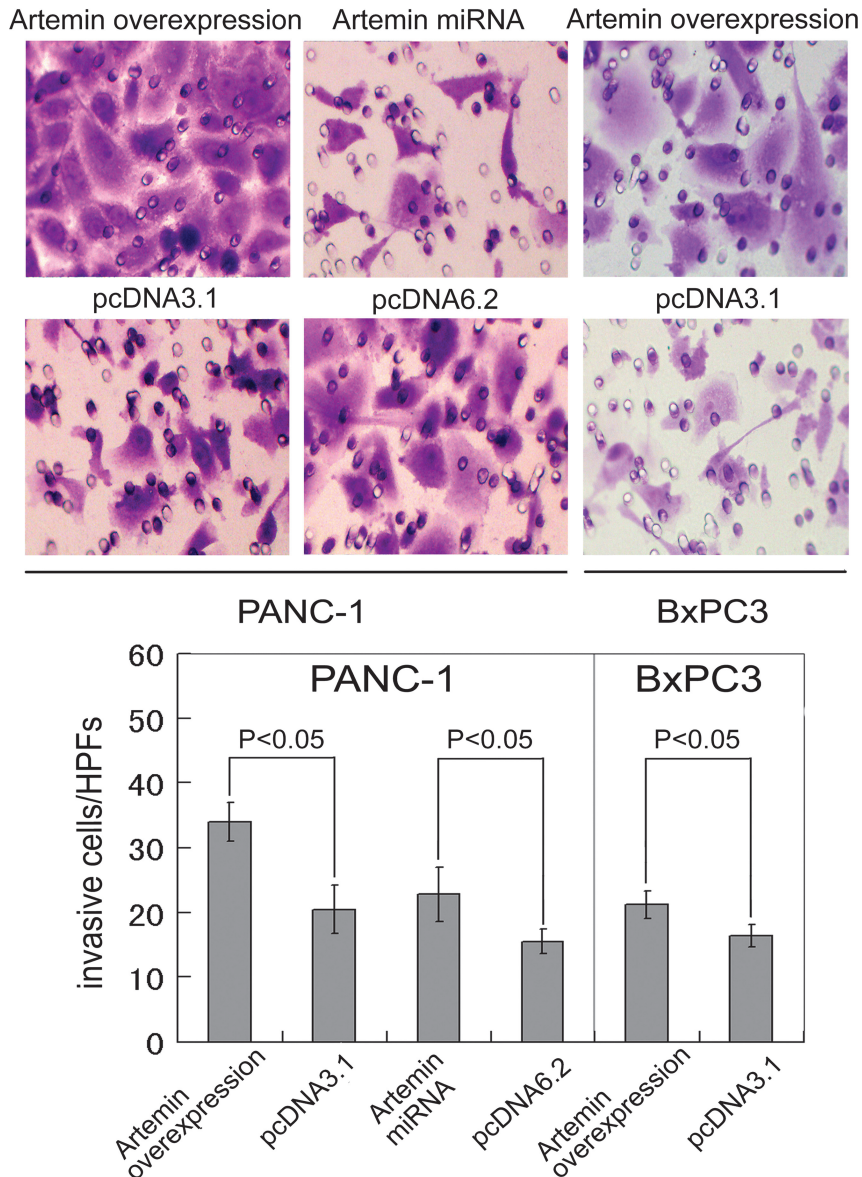
FIGURE 5. Effect of Artemin on the invasiveness of pancreatic cancer cells. Number of invading cells was significantly larger in Artemin overexpressing PANC-1 and BxPC3 than in their respective control groups ($P < 0.05$) and less in the Artemin depletion group than in the empty vector control group ($P < 0.05$).

cancer cell invasion. The RNAi-induced inhibition of Artemin decreased the invasive potential of cancer cells (Fig. 5).

Induced Differentiation Test of PC12 Cells

Undifferentiated PC12 cells were oval, small polygonal or short spindle, and loosely attached undergoing rapid proliferation. These cells could reach a confluence of 100% in 10 days when they were seeded at a density of 1×10^3 cells/well (Fig. 6A). The cell proliferation was inhibited when the PC12 cells were cultured with the culture supernatant of the Artemin-overexpression PANC-1 cell line. Most of the cells were then shaped into long spindles as they increased in size and appeared polygonal in shape with a slender projection similar to the neurons in the neural synapse (Fig. 6B). Fewer neuronlike cells could be seen in the control group (Fig. 6C). There were groups of oval or short polygonal

undifferentiated cells and few neuronlike PC12 cells in the Artemin RNAi group (Fig. 6D), different from its control (Fig. 6E).

Tumorigenesis in Nude Mice

One mouse died in 1 to 2 days after P6.2 and Ai groups were vaccinated. Five mice in the Ai group were dissected; among these mice, three showed visible tumors, and one exhibited microscopically identified minimal tumor lesions beneath the pancreatic capsule (Fig. 7A). In 1 case, a microscopic necrotic lesion was found in the implanted site of the pancreas (Fig. 7B), but no tumor was found. The tumor-forming rate was 80% in the Ai group; in other groups, this rate was 100%.

Tumors were located in the body and tail of the pancreas exhibiting irregular shapes and inconspicuous capsules. The cut
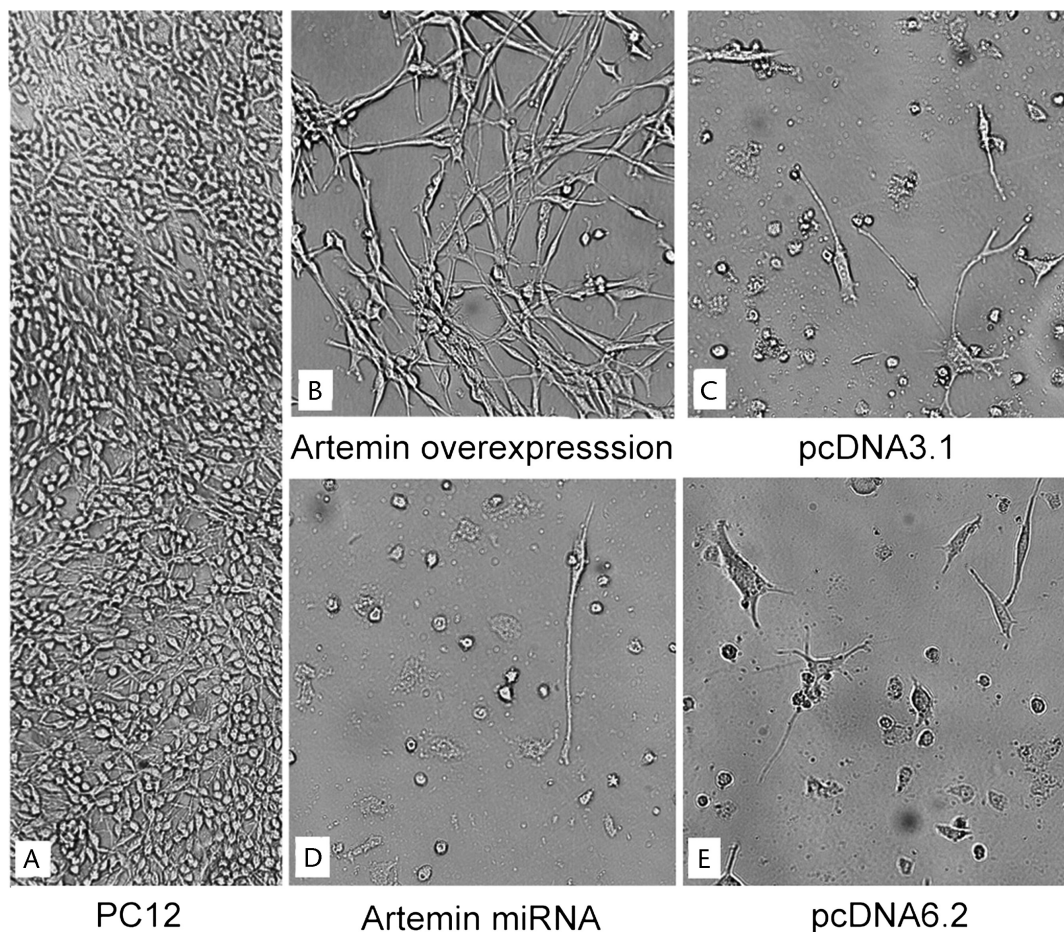
FIGURE 6. Induced differentiation test of PC12 cells. A, PC12 cell for blank control was undifferentiated and fully seeded in 6-well plates on the 10th day. B, PC12 cells cultured with supernatant of Artemin-overexpressed PANC-1 cells stopped growth and showed significant neuronlike differentiation. C, PC12 cells cultured with supernatant of the empty vector pCDNA3.1 control cells showed a few neuronlike differentiation cells. D, PC12 cells cultured with supernatant of Artemin-depleted PANC-1 cells showed inconspicuous neuronlike differentiation. E, PC12 cells cultured with supernatant of the empty vector pCDNA6.2 control showed a few neuronlike differentiation cells.

section was off-white; solid and hard; and partly adherent to the liver, spleen, kidney, abdominal muscles, or diaphragm.

Tumors grew in sheets with few stroma. Most cases in the Artemin RNAi group were restricted with clear borders, and a pseudocapsule could be seen (Fig. 7C); the tumors in the Artemin overexpression group lacked restrictive borders and invaded the peripheral pancreatic tissues in a crablike pattern (Fig. 7D). The incidence of tumor thrombus (Fig. 7E) and nerve invasion (Fig. 7F) in the Artemin overexpression group were higher than that in the Artemin RNAi group ($P < 0.05$), but no significant difference was found comparing with their respective control groups ($P > 0.05$). Approximately 50% (3/6) of the Artemin overexpression group exhibited lymphatic metastasis, whereas no metastasis was observed in the Artemin RNAi group; however, this result is not supported by statistical evidence. Tumor size and pathology features of each group are compared in Table 3.

The IHC revealed that GAP-43 was positively expressed in the stromal nerve fibers of tumor and peritumoral tissues (Figs. 7G, H). By contrast, GAP-43 was negatively expressed in the intestinal mysenteric plexus, which was located distantly from the tumors. The Artemin overexpression group expressed the highest GAP-43-positive stromal nerve fibers, whereas the Artemin RNAi group expressed the least GAP-43-positive stromal nerve fibers among the groups (Table 3). However, no statistical evidence was found

comparing with their respective control groups ($P > 0.05$). This result may be attributed to the small sample size. The maximum area of nerve fibers was approximately equal in all of the groups.

DISCUSSION

The susceptibility of PNI in pancreatic adenocarcinoma may be related to at least 2 conditions: (1) abundant nerve fibers found in tumor stroma provide the material basis of cancer cell PNI, and (2) enhanced cancer cell invasiveness contributes to cancer cells invading the nerve sheath and spreading along the nerves. Our study showed that Artemin may be involved in promoting nerve growth and pancreatic cancer cell invasion in vitro, in pancreatic carcinoma tissues, and in animal models. We also investigated the potential therapeutic value of Artemin expression as a target in pancreatic adenocarcinoma.

Pathological findings included the evident hypertrophy and hyperplasia of nerve fibers as well as neural remodeling in the stroma of pancreatic adenocarcinoma. The GAP-43 is a nerve-specific phosphorylated protein and up-regulated in neurons in response to nerve destruction. This protein influences the budding, growth, and formation of synapses. The GAP-43 can be expressed in hypertrophic nerve fibers in pancreatic adenocarcinoma and chronic pancreatitis but not in benign tumors or other
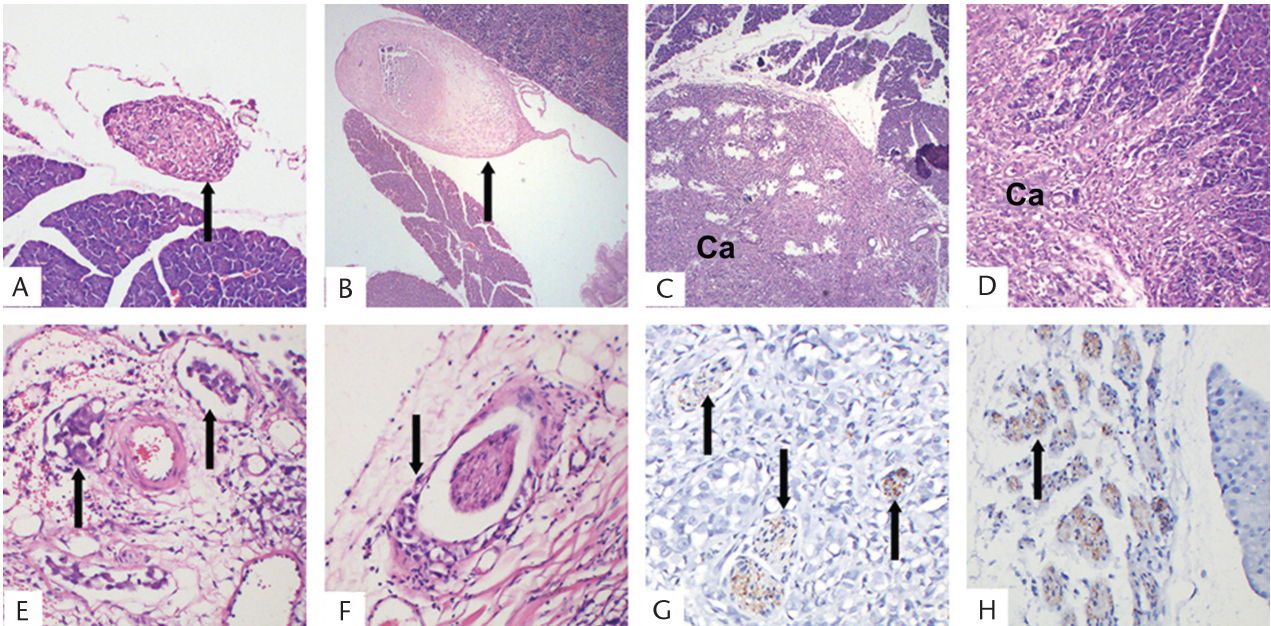
FIGURE 7. Microscopic finding of transplanted tumors in nude mice. A, Minimal tumor lesions beneath the capsule of the pancreas in the Artemin RNAi group (hematoxylin-eosin staining (HE), 40×). B, Fibrotic lesions in the implanted site of the pancreas in the Artemin RNAi group (HE, 40×). C, Clear borders between tumors and normal pancreatic tissues in the Artemin RNAi group. A pseudocapsule could also be observed (HE, 40×). D, Crablike invasion of the normal pancreatic tissues in the Artemin overexpression group (HE, 40×). E, Tumor thrombus in the vessels of the Artemin overexpression group (HE, 100×). F, Tumor cells surrounded the nerve sheath in the Artemin overexpression group (HE, 100×). G, Positive expression of GAP-43 in the stromal nerve fibers of tumors by IHC in the Artemin overexpression group (IHC, 100×). H, Increase in the stromal nerve fibers of peritumoral pancreatic tissues could be observed in the Artemin overexpression group, showing that GAP-43 was positively expressed (IHC, 100×).

lesions.[10] Thus, GAP-43 is an indicator of nerve growth and remodeling. We determined the number of GAP-43-positive cells and area of intrapancreatic nerve fibers by conducting IHC combined with the Image J software. The mean density and maximum area of nerve fibers in the Artemin-positive group were higher than those in the Artemin-negative group. This result indicated that the overexpression of Artemin in pancreatic cancer cells may be related to the hypertrophy and hyperplasia of stromal nerve fibers in tumors. This result is also consistent with a previous study[10] and may be used as the basis of neural invasion of pancreatic adenocarcinoma.

An induced differentiation test with PC12 cells was performed to prove the neurotrophic effect of pancreatic cancer cell by paracrine Artemin protein. The PC12 is an adrenal pheochromocytoma cell line. The undifferentiated cells showed oval, polygonal, or short spindle shapes; such cells exhibited neuron-like differentiation as induced by neural growth factors, and these factors can be used to identify the activity of the neurotrophic factor in vitro.[11] We used the supernatants of Artemin-overexpressed pancreatic cancer cells to culture PC12 cells and found that Artemin significantly induced cancer cell differentiation. Thus, pancreatic cancer cells could be neurotrophic by secreting Artemin. Although this effect could have possibly resulted from the combination of several other factors, this process was markedly attenuated in the control group, which thus demonstrated the neurotrophic function of Artemin. Ceyhan et al[12] showed that the extractions of pancreatic tumor tissues and peritumoral tissues can promote the formation of the neural network in the intestinal plexus of mice in vitro; this formation can be inhibited by an Artemin antibody. Thus, pancreatic tumor cells possibly perform a neurotrophic function by overexpressing neurotrophic factors, such as Artemin. Moreover, the neurotrophic effects of pancreatic carcinoma can extend into the peritumoral pancreatic areas without neurocancer interactions. However, whether Artemin in the extractions is obtained from pancreatic cancer cells or stroma neurons remains unclear. Our in vitro experiment further demonstrated that Artemin secreted by pancreatic cancer cells elicits a neurotrophic effect. Abundant

TABLE 3. Comparison of Tumorigenesis in Nude Mice Groups

Group	Tumor Diameter, mm	Peripheral Organ Invasion	Tumor Thrombus	Lymphatic Metastasis	Nerve Invasion	Amount of Nerve Fibers, /cm^2	Maximal Area of Nerve Fibers, ×10^{-4} mm^2
pCDNA3.1	11.33 (1.366)	4/6	2/6	1/6	2/6	17.67 (8.017)	84.33 (60.474)
Overexpression	14.00 (2.366)*	6/6	4/6	3/6	4/6	34.33 (28.190)	93.00 (90.322)
RNAi	3.06 (3.011)*†	1/5†	0/5†	0/5	0/5†	10.60 (14.398)	81.48 (92.277)
pCDNA6.2	8.80 (2.950)	3/5	2/5	1/5	0/5	16.00 (7.000)	74.00 (81.389)

*$P < 0.05$, compared with the control group. †$P < 0.05$, compared with the upper group.

GAP-43-positive nerve fibers could be observed in the transplanted tumor tissues and in the stroma surrounding the tumors of nude mouse transplantation models. This result was commonly observed in the Artemin overexpression group but rarely in the Artemin RNAi group. This result further indicated that the neurotrophic effects of pancreatic carcinoma were observed in the peritumoral areas. However, no statistical difference in the number and area of nerve fibers was found between experimental groups and their respective control groups possibly because of a small sample size used in this study.

Artemin has the ability to induce the proliferation of neuroblasts.[13] However, in nonneurogenic tumors, Artemin could promote the invasiveness of cancer cells. Many neural growth factors could promote the growth and invasiveness of tumors by participating in the interaction of tumor cells and nerve fibers. These neurotrophic factors are derived from nerve tissues of the pancreas and secreted by pancreatic cancer cells. Four members, including GDNF, neurturin, Artemin, and persephin, of the GDNF family could be expressed in pancreatic adenocarcinoma. Moreover, ret proto-oncogene (RET), the common receptor of these 4 members, is the product of the oncogene c-RET and can be expressed in most pancreatic cancer cell lines.[14] In vitro experiments[15] have shown that GDNF can promote the invasiveness of pancreatic cancer cells and enhance chemoattractant effect. Artemin is a member of the GDNF family and can transfer signals to cells via the RET receptor. However, the effect of Artemin on pancreatic cancer cells remains unclear and has not been proven in vivo.

The earliest description of the relationship between Artemin and pancreatic adenocarcinoma was published in 2005. Ito et al[14] found that the positive expression rate of Artemin in pancreatic adenocarcinoma is 75%; Artemin is positively expressed in the cytoplasm of cancer cells and the stromal nerves of tumors as well as in the ductal epithelium and the nerves in peritumoral tissues. In 2006, Ceyhan et al[16] found that the expression levels of Artemin and its receptor GFRα3/RET were higher in pancreatic adenocarcinoma than in normal pancreatic tissues. In our study, the expression of Artemin protein and its positive rate in IHC were consistent with those reported in literature. Moreover, the positive rate is related to lymphatic metastasis and nerve invasion. Thus, we hypothesized that Artemin may enhance the invasiveness of pancreatic cancer cells to facilitate invasion of the vessels or neural sheaths.

This hypothesis was further validated in in vitro experiments. Artemin expression was found in 6 kinds of pancreatic cancer cell lines by Western blot. A higher expression was found in cell lines with high invasive potential such as SW1990 and AsPC-1 compared with that in cell lines with low invasive potential such as BxPC3. It indicated that Artemin may play an important role in cancer cell invasion. The PANC-1 cell line was transfected with Artemin-overexpression plasmid and Artemin RNAi plasmid, and BxPC3 cell line was transfected with Artemin-overexpression plasmid. The results showed that neither of these 2 plasmids could change the growth rate of cancer cells. Flow cytometry results indicated that Artemin failed to accelerate the proliferation of cancer cells as observed in the cell cycles; this result may be attributed to a different molecular basis or state of pancreatic cancer cells from neurogenic cells. No correlation was found between Artemin expression and cell apoptosis. However, the overexpression of Artemin could increase the colony-forming rate, migration rate, and invasion proportion of pancreatic cancer cells. Ceyhan et al[16] seeded pancreatic cancer cells with recombinant human Artemin protein and found that the proportion of invaded cancer cells increases in the Matrigel invasion assay. In other epithelial cancers, such as non–small-cell lung cancer,[9] breast cancer,[17] and endometrial cancer,[7] Artemin elicits similar effects, including promotion of survival, colony formation, migration, and invasion of tumor cells.

Although in vitro RNAi could inhibit the effects of Artemin overexpression, the lack of subtle interaction between cancer cells and the surrounding microenvironment raised the question of whether this inhibition can proceed even in a pancreatic microenvironment in vivo. Artemin elicits similar effects in animal models of breast cancer,[17] endometrial cancer,[7] and non–small-cell lung cancer,[9] in which the overexpression of Artemin increases the volume and enhances the invasiveness and metastasis of transplanted tumors. Our study is the first to demonstrate the same effects by using nude mouse pancreatic tumor transplantation models. Artemin-overexpressed pancreatic cancer cells showed greater invasiveness by deeply invading the surrounding pancreatic tissues and peripheral organs, such as the spleen, liver, kidneys, intestinal walls, and diaphragm. The incidence rates of intravascular thrombus, nerve invasion, and lymphatic metastasis could also increase. Moreover, the Artemin RNAi plasmid could significantly decrease the area of transplanted tumors and inhibit these effects. This result also showed that Artemin could promote pancreatic cancer invasion.

Artemin elicits potential therapeutic effects on neural degeneration, injury,[18] and neurogenic pain.[19] However, Artemin functions as an oncogene that affects biological behaviors to promote the invasiveness of cancer cells[7,17,20] in many tumors, mediate the drug resistance of estrogen receptor-positive breast cancer cells,[8] and lower the sensitivity of endometrial cancer cells to taxol and doxorubicin.[21] These results suggested that the inhibition of Artemin could be a therapeutic target in several tumors. The RNAi could induce defective posttranscriptional genetic phenotypes; RNAi has been successfully applied in the study of gene functions as well as the upstream and downstream relationships of signaling pathways.[22] Our study is the first to perform RNAi to inhibit Artemin expression in pancreatic cancer cells. Artemin RNAi effectively suppressed tumor growth and invasion in vitro and in animal models. Therefore, Artemin could be used as a potential therapeutic target of pancreatic adenocarcinoma.

CONCLUSIONS

This study proved that Artemin expressed in pancreatic cancer cells exhibited the following: (1) promote the invasiveness of cancer cells and (2) function as a neurotrophic factor via paracrine signaling. This study is the first to identify these effects in nude xenograft models. This study also showed that the inhibition of Artemin expression in pancreatic cancer cells by RNAi could inhibit cancer cell invasion both in vitro and in vivo. Therefore, Artemin could be used as a potential therapeutic target of pancreatic adenocarcinoma.

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