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Upregulation of transgelin is an independent factor predictive of poor prognosis in patients with advanced pancreatic cancer

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Transgelin is a known actin-binding protein, which plays a role in regulating the functions of smooth muscle cells or fibroblasts. Recent evidence indicates that transgelin is involved in diverse human cancers, yet its role in pancreatic cancer remains unclear. We therefore evaluated the expression characteristics and function of transgelin in pancreatic cancer. Immunohistochemical analysis of benign (n = 30 patients) and malignant (n = 114 patients) pancreatic ductal cells showed significantly higher transgelin staining in malignant cells. Lymph node metastasis (P = 0.026) and diabetes (P = 0.041) were shown to significantly correlate with transgelin protein expression. Patients with high transgelin expression showed a shorter 5-year overall survival and a lower tumor-specific survival than those with low transgelin expression. Multivariate analysis revealed that transgelin was an independent factor affecting pancreatic tumor-specific survival (P = 0.025). *In vitro*, RNA interference-mediated transgelin knockdown resulted in inhibition of pancreatic cancer cell proliferation, migration and invasion. Depletion of transgelin expression could suppress pancreatic tumorigenicity and tumor growth *in vivo*, and produce enhanced cytotoxic effects of gemcitabine on pancreatic cancer cells both *in vitro* and *in vivo*. Our results indicate that transgelin plays a promoting role in tumor progression, and appears to be a novel prognostic marker for advanced pancreatic cancer. (*Cancer Sci* 2013; 104: 423–430)

Pancreatic cancer is one of the most aggressive human cancers, with a 5-year survival rate of approximately 5%.[1] The disease is characterized by rapid tumor spread and dismal prognosis. By the time of diagnosis, <15% of patients can be offered a potential curative treatment, mainly due to the high frequency of local or regional spread and distant metastasis.[2] Recent studies have revealed a close relationship between actin-associated protein and pancreatic cancer.[3] Tumor invasion and metastasis are critically dependent on dynamic alterations in the organization of the actin cytoskeleton, and dysregulation of actin-based motility is a prominent factor in cell transformation.[4] Therefore, actin-associated protein has attracted increasing attention due to its potential contribution to the pathogenesis of pancreatic cancer.

Transgelin, which is also referred to as smooth muscle protein 22α, is a shape-change sensitive actin cross-linking/gelling protein found in fibroblasts and smooth muscle.[5] It is a member of the calponin family and is localized to the cytoskeletal apparatus.[6] Transgelin can associate with actin and has long been known as an early marker of smooth muscle differentiation.[7] It is involved in podosome formation in smooth muscle cells and regulates the development and contractile function of many cells.[8] Recently, transgelin was suggested to be involved not only in regulating the functions of smooth muscle cells or fibroblasts, but also in carcinogenesis in various tissues;[9–11] however, its expression pattern and biological functions are not consistent in all tumors, not even in the same tumors. Published studies report that loss of transgelin is a characteristic signature of colon and prostate carcinogenesis and its restoration suppresses colon tumorigenity *in vivo* and *in vitro*.[9,10,12,13] In contrast, overexpression of transgelin in gastric carcinoma[14] and node-positive colorectal cancer[15] are demonstrated in other reports. Further investigation in colorectal cancer cell lines has revealed roles for transgelin in promoting invasion, survival and resistance to anoikis.[15] Paradoxically, there is previous evidence that transgelin is both a tumor suppressor and a variable tumor biomarker, depending on the tumor type, stage and experimental model.[10]

Although several proteomic investigators have revealed the marked upregulation of transgelin in human pancreatic cancer tissues,[16–18] little is known about its expression characteristics and functions in relation to the aggressive disease. Thus, in the present study, we investigated the expression of transgelin in pancreatic cancer tissues by immunohistochemical analysis, and compared it with the clinicopathological factors of pancreatic cancer and patient prognosis. In addition, the roles of transgelin in pancreatic cancer cell growth and tumorigenicity were examined, under both *in vitro* and *in vivo* conditions.

Materials and Methods

Patients and tissue specimens. Formalin-fixed paraffin-embedded tissue blocks containing 114 pancreatic cancer and 30 adjacent tumor-free tissues were subjected to immunostaining. All of the patients underwent pancreatectomy in the Department of Surgery, the First Affiliated Hospital of Zhengzhou University, between June 2002 and January 2007. All the specimens in this study were determined as pancreatic ductal adenocarcinoma by histologic examination. There were 79 male and 35 female patients, with ages ranging from 31 to 79 years (median, 64 years). The median follow-up data was 15.0 months (range 0.2–60 months) and 111 out of 114 patients died during the follow-up period. For the analyses of overall survival, each patient's time began on the date of diagnosis and ended on the date of death or on the date last seen alive. Tumor-specific survival was also evaluated. To calculate

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tumor-specific survival, deaths clearly documented to be resulting from non-cancer causes (e.g. severe infection and treatment side effects) were treated as withdrawals in the tumor-specific survival analyses. The pathologic tumor staging was determined according to the 2009 Union Internationale Contre le Cancer/American Joint Committee on Cancer TNM staging system. Diabetes was diagnosed following the American Diabetes Association criteria.[19] This work was done with the approval of the Ethics Committee of the First Affiliated Hospital of Zhengzhou University, and informed consent was obtained from all patients.

Cell lines and cultures. Human pancreatic ductal cancer cell lines SW1990 and BxPC3 were originally obtained from the American Type Culture Collection (Rockville, MD, USA). All cells were routinely maintained in DMEM (Life Technologies, Grand Island, NY, USA) containing 10% FBS (Life Technologies) in a humidified incubator at 37°C with an atmosphere of 5% CO_2.

Immunohistochemical staining. Immunohistochemical staining of 3 μm paraffin sections for transgelin protein and proliferation of cell nuclear antigen (PCNA) protein were performed with an LSAB kit (DAKO, Marseille, France), using antihuman transgelin antibody (dilution 1:50; Proteintech Group, Chicago, IL, USA) and antihuman PCNA antibody (dilution 1:500; Cell Signal, Boston, MA, USA). The sections were incubated in 3, 3′-diaminobenzide tetrahydrochloride with 0.05% H_2O_2 for 3 min and then counterstained with Mayer's hematoxylin. The staining of transgelin was scored as the product of the staining intensity (on a scale of 0–3: negative = 0, weak = 1, moderate = 2 and strong = 3) and the percentage of cells stained (on a scale of 0–3: 0 = zero, 1 = 1–25%, 2 = 26–50% and 3 = 51–100%), resulting in scores on a scale of 0–9.[20] We used the following criteria to assess the immunohistochemical results: high-level expression of transgelin was defined when the score of transgelin staining was 3 or more; low-level expression was defined when the score of transgelin staining was <3.

Western blot analysis. Cells were lysed in 2× Laemmli buffer containing a cocktail of proteinase inhibitors (Bio-Rad, Hercules, CA, USA). The protein extraction method was as described in a previous study,[21] boiled for 10 min at 100°C, and then resolved by SDS-PAGE on a 12% gel. Subsequently, the proteins were electrophoretically transferred to a nitrocellulose membrane. The membranes were blocked with antihuman transgelin antibody (dilution 1:100) and antihuman actin antigen (dilution 1:2000; Oncogene, San Diego, CA, USA), followed by incubation with HRP-conjugated secondary antibody. The protein signals were detected by luminal detection reagent (Santa Cruz Biotechnologies, Santa Cruz, CA, USA).

Plasmid construction. To construct vectors for expressing transgelin-shRNA, the pGCsi-H1 plasmid (GeneChem, Shanghai, China), which contains a green fluorescent protein gene under a separate promoter for tracking the transfection efficiency, was digested with *Bam*HI and *Hin*dIII. Three chemically synthesized oligonucleotides encoding transgelin-shRNA that included a loop motif were inserted downstream of the H1 promoter of the plasmid using a DNA ligation kit, and cloned. The targeting sequences of human transgelin (GeneBank accession: NM 003186) are 5′-CCAAAATCGAGAAGAAGTA-3′, 5′-CCAGACTGTTGACCTCTTT-3′ and 5′-AGAAAGCGCAGGAGCATAA-3′, corresponding to the coding regions of positions 168–186, 457–475 and 582–600, respectively. The pGCsi-H1/negative vector was used as the negative control in all experiments. The accuracy of sequences inserted into the recombinants was verified by restriction enzyme analysis and sequencing.

Plasmid transfection and selection of stable transfectants. A day before transfection, SW1990 and BxPC3 cells were plated in six-well plates with 1×10^5 cells/well using culture medium without antibiotics. The cells were transfected with 3.0 μg/well of different pGCsi-H1-transgelin or pGCsi-H1/negative plasmid using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. Four hours after transfection, the cells were passaged at a 1:15 dilution (v/v) and cultured in medium supplemented with Geneticin (G418; Life Technologies) at 800 μg/mL for 4 weeks. Stably transfected clones were picked and maintained in medium containing 350 μg/mL G418. Two knockdown clones of each cell line were selected for *in vitro* experiments.

Cell growth assay. Cell growth was evaluated by WST-8 (2-methoxy-4-nitrophenyl-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2Htetrazolium, monosodium salt) cleavage assay (Cell Counting Kit-8; Dojindo, Kumamoto, Japan). SW1990 and BxPC3 cells (5×10^3) stably transfected with pGCsi-H1-transgelin (SW1990/shTrans-1, SW1990/shTrans-2 and BxPC3/shTrans-1 and BxPC3/shTrans-2) or pGCsi-H1/negative (SW1990/shControl and BxPC3/shControl) were plated in 96-well microplates. Cells were incubated in serum-free DMEM medium for 24, 48 and 72 h. After addition of 10 μL of the Cell Counting Kit-8 reagent followed by 3 h incubation, the plates were read at 450 nm in a spectrophotometer (Tecan Safire2, San Jose, CA, USA).

Colony formation assay. Cells (3×10^3) were trypsinized to single-cell suspension and then plated in triplicate onto six-well plates in complete culture medium containing 0.3% agar on top of 0.6% agar in the same medium. The plates were incubated at 37°C in 5% CO_2 for 14 days. Colonies were fixed with 70% ethanol and stained with 0.2% crystal violet. Colonies with diameters larger than 50 μm were counted at 14 days after plating.

Cell migration and invasion assays. See "Document S1."

***In vivo* xenograft experiment and tumorigenesis assay.** Balb/c nude mice were maintained under specific pathogen-free conditions in the Shanghai Experimental Animals Centre of the Chinese Academy of Sciences. SW1990, SW1990/shControl and SW1990/shTransgelin (SW1990/shTrans-1) cells were harvested and resuspended in PBS, respectively, and then 5×10^6 cells in 100 μL PBS were injected s.c. into the right flank of a 4-week-old Balb/c nude mouse. Six mice were included in each group; namely, SW1990, SW1990/shControl, SW1990/shTransgelin, SW1990/shControl + gemcitabine (GEM; Eli Lilly, Indianapolis, IN, USA), and SW1990/shTransgelin + GEM. Seven days after implantation, mice in the SW1990/shTransgelin + GEM group and the SW1990/shControl + GEM group received an i.p. injection of 50 mg/kg GEM twice a week, respectively. To standardize the experiments, mice in the SW1990, SW1990/shControl and SW1990/shTransgelin group were administered equal volumes of PBS at the same time point. Tumor size was measured using a caliper and was calculated according to the formula $AB^2/2$, where A is the longest diameter and B is the shortest diameter of the tumor. Animals were killed 35 days after cell implantation. Tumors were weighed and a portion of each was placed in 4% paraformaldehyde. All protocols were approved by the Institutional Animal Care and Use Committee of Zhengzhou University.

Statistical analysis. Values are presented as mean ± SEM. Statistical analyses were conducted using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Statistical differences between groups were assessed by the unpaired two tailed *t*-test or ANOVA test, or Mann–Whitney *U*-test when data were not parametric. Multivariate logistic regression analysis was performed to identify independent risk factors for the expression of transgelin protein. Survival curves were drawn according to the Kaplan–Meier method and differences between the curves were

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analyzed by applying the log-rank test. Multivariate Cox regression analysis was used to analyze the independent prognostic factors of survival. P-values < 0.05 were considered statistically significant.

Results

Transgelin is upregulated in pancreatic cancer specimens. Immunohistochemical analysis for transgelin was carried out in 114 pancreatic tumors and 30 adjacent normal pancreatic tissues. Transgelin immunostaining was predominantly positively identified in the cytoplasm, scattered throughout the neoplasm. Compared with malignant ductal cells (Fig. 1b–d), transgelin immunostaining in benign ducts was negative or relatively weak (Fig. 1a). In addition, transgelin immunostaining was much stronger in most pancreatic cancer specimens from patients with diabetes (Fig. 1c,d) compared with those without diabetes (Fig. 1b). According to the criteria of semi-quantitative assessment employed, transgelin was highly expressed in 86 (75.4%) of 114 pancreatic cancers and 11 (36.7%) of 30 normal pancreatic tissues. Statistical analysis of transgelin staining scores confirmed increased staining in malignant cells compared with benign ductal cells (Fig. 1e, Mann–Whitney U-test, $P < 0.01$).

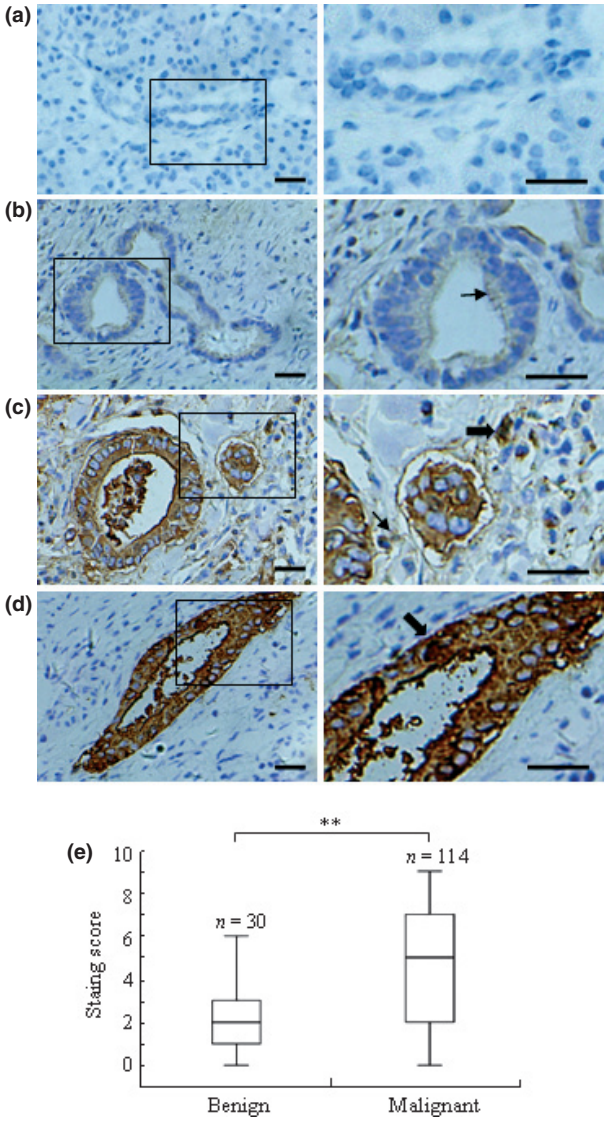

Fig. 1. Immunostaining for transgelin protein in human pancreatic tissues. Histopathologic sections of pancreas were stained with anti-transgelin antibody. The staining of immunoreactivity was expressed as a product of the intensity and the proportion of cells staining positive. The positive staining of pancreatic ductal cells was expressed as yellow–brown granules with weak (thin arrows) to moderate–strong (thick arrows) intensity. Transgelin immunoreactivity was readily detected in the cytoplasm and occasionally in the nucleus. (a) Normal ducts showing negative transgelin expression. Malignant ductal cells are shown with (b) weak, (c) moderate and (d) strong transgelin staining. (e) Comparison between staining score distributions in malignant and benign cells using the Mann–Whitney U-test. $^{**}P < 0.01$. Original magnification, ×200 (left column), bars = 50 μm.

High levels of transgelin were associated with reduced patient survival. We next analyzed the relationship between transgelin protein expression and clinicopathologic factors (Table 1). In univariate analysis, lymph node metastasis and diagnosis of diabetes pre-surgery had some correlation with transgelin protein expression. There was no significant correlation between transgelin expression and other factors, such as age, gender, histologic differentiation and local invasion. Among these factors, lymph node metastasis (odds ratio [OR]: 2.958, 95% confidence interval [CI]: 1.138–7.690) and diagnosis of diabetes pre-surgery (OR: 2.699, 95% CI: 1.041–6.998) were proven to be independent predictors of high transgelin protein expression by multivariate logistic regression analysis (Table 2).

The 5-year overall survival rate was 2.6% (3/114). There was a statistically significant difference in the 5-year survival

Table 1. Correlation between transgelin expression and clinicopathological parameters of patients with pancreatic cancer

Characteristics	All cases	Transgelin level		χ^2	P-value
		High	Low		
Total participants	114	86	28		
Age at surgery (years)					
<60	34	25	9	0.095	0.758
≥60	80	61	19		
Gender					
Male	79	58	21	0.567	0.451
Female	35	28	7		
Tumor grade					
Poorly	51	37	14	2.747	0.253
Moderately	50	41	9		
Well	13	8	5		
Tumor stage					
I–II	53	39	14	0.184	0.668
III–IV	61	47	14		
Tumor size					
≤20 mm	29	18	11	3.752	0.053
>20 mm	85	68	17		
Lymph node metastasis					
Positive	55	47	8	5.754	0.016
Negative	59	39	20		
Vascular invasion					
Positive	52	39	13	0.010	0.921
Negative	62	47	15		
Perineural invasion					
Positive	53	44	9	3.072	0.080
Negative	61	42	19		
Resection margin					
Involved	19	16	3	0.464	0.496
Clear	95	70	25		
Diabetes					
With	54	46	8	5.260	0.022
Without	60	40	20		

Table 2. Multivariate analysis for factors shown to correlate to transgelin expression by multivariate logistic analysis

Factor	Odds ratio (95% CI)	*P*-value
Tumor size (> 20 mm)	0.445 (0.144–1.368)	0.158
Lymph node metastasis (yes)	2.958 (1.138–7.690)	0.026
Diabetes (yes)	2.699 (1.041–6.998)	0.041

CI, confidence interval.

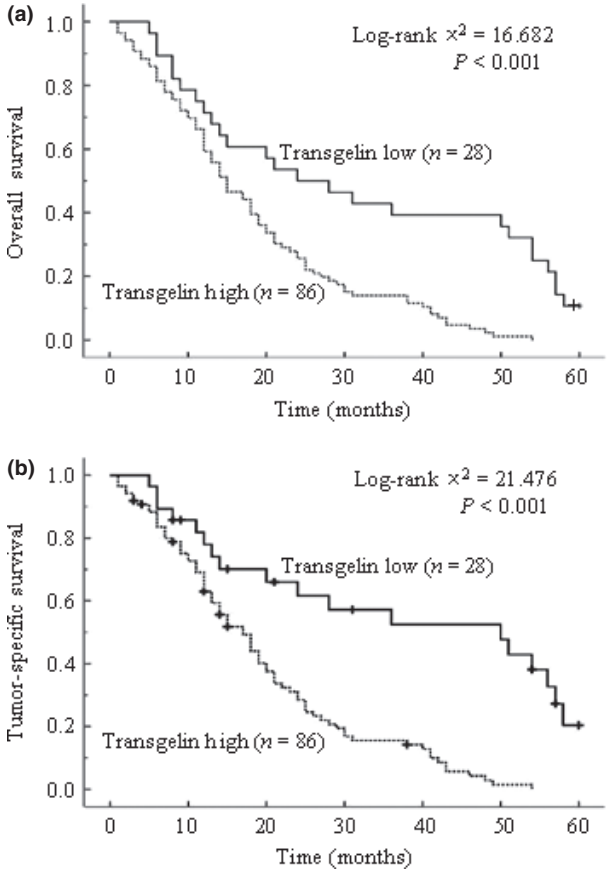

Fig. 2. Kaplan–Meier analysis of overall survival and tumor-specific survival depending on transgelin expression in pancreatic cancer. Patients with high-transgelin (staining score $\geq$3) tumors had a shorter overall survival (a) ($P < 0.001$) and a shorter tumor-specific survival (b) ($P < 0.001$) than patients with low-transgelin (staining score <3) tumors.

between transgelin-high and transgelin-low tumors: 0% *vs* 10.7% ($P < 0.001$; Fig. 2a). During this period, 97 patients experienced metastatic recurrence and died of pancreatic cancer directly, 14 died of non-cancer causes, such as side effects from treatment. The 5-year tumor-specific survival rate was significantly lower in patients with high transgelin expression: 0% *vs* 35.7% ($P < 0.001$; Fig. 2b). Multivariate analysis using the Cox proportional hazard model indicated that, apart from tumor staging (HR = 5.103, 95% CI: 2.696–9.660) and lymph node metastasis (HR = 5.928, 95% CI: 3.161–11.118), high expression of transgein (HR = 1.898, 95% CI: 1.085–3.319) significantly correlated with poor tumor-specific survival and was an independent prognostic factor for pancreatic cancer (Table 3).

Suppression of transgelin expression resulted in impaired cell growth and tumorigenicity. Western blot analysis indicated that

Table 3. Cox proportional hazards model analysis of prognostic factors in patients with pancreatic cancer (n = 114)

Factor	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
Age	1.068 (0.667–1.712)	0.784	NA	NA
Gender	0.705 (0.449–1.106)	0.128	NA	NA
Tumor staging	4.500 (2.271–8.916)	<0.001	5.103 (2.696–9.660)	<0.001
Tumor size	1.487 (0.945–2.340)	0.086	NA	NA
Lymph node status	6.398 (3.368–12.154)	<0.001	5.928 (3.161–11.118)	<0.001
Vascular invasion	1.164 (0.767–1.765)	0.476	NA	NA
Perineural invasion	1.097 (0.704–1.710)	0.683	NA	NA
Resection margin	1.730 (1.004–2.981)	0.048	NA	NA
Diabetes	1.218 (0.765–1.941)	0.406	NA	NA
Transgelin expression	1.878 (1.043–3.382)	0.036	1.898 (1.085–3.319)	0.025

NA, not applicable.

pGCsi-H1-168 plasmid was the most effective construct in knockdown transgelin expression (Fig. S1). Hence, two stable transfected clones of each cell line containing pGCsi-H1-168 plasmid were used for further study (Fig. 3a). In both SW1990 and BxPC3, shTransgelin treatment showed significantly lower WST-8 cleavage levels than cells transfected with control plasmid at each time point of incubation (Fig. 3b,c). We then evaluated clonogenic survival by plating cells at low density and scoring for colony formation after 14 days (Fig. 3d). Knockdown othe f transgelin inhibited the clonogenic survival of SW1990 by approximately 65.2% and 67.8% and BxPC3 by approximately 61.1% and 64.9% (Fig. 3e). Compared with their controls, cells transfected with shTransgelin exhibited not only a lower amount but also a smaller size of colonies.

Suppression of transgelin expression resulted in impaired cell motility. In cell migration assay using Millicell inserts (Fig. 4a), cell numbers translocating across the microporous membranes after shTransgelin treatment were significantly decreased by an average of 58.7% and 63% in SW1990 cells and 67.5% and 65% in BxPC3 cells, respectively, when compared with control treatments after 24 h of incubation (Fig. 4b). Similar changes were observed in cell invasion assay through Matrigel basement membrane matrix (Fig. 4c). shTransgelin treatment significantly reduced the invasion in SW1990 cells by 58.1% and 54.8% and in BxPC3 cells by 68.0% and 64% (Fig. 4d). Cell migration was also detected using the wound healing assay (Fig. 4e). After 48 h of incubation, shTransgelin treatment impaired cell migration by an average 49.5% and 51.6% when compared with controls in SW1990 cells ($P < 0.01$; Fig. 4f). Treatment of BxPC3 cells with shTransgelin reduced migration by 48.2% and 44.6% compared with controls ($P < 0.01$; Fig. 4f). These results strongly indicate that transgelin could increase the migration and invasion ability in pancreatic cancer cells *in vitro*.

Suppression of transgelin expression inhibits tumorigenicity and tumor growth *in vivo*. We further analyzed the role of transgelin in tumorigenicity and tumor growth *in vivo* using an immunodeficient nude mouse model. Because two clones of each cell line gave similar results in our cell growth, motility and invasion assays, we selected transgelin knockdown clone 1

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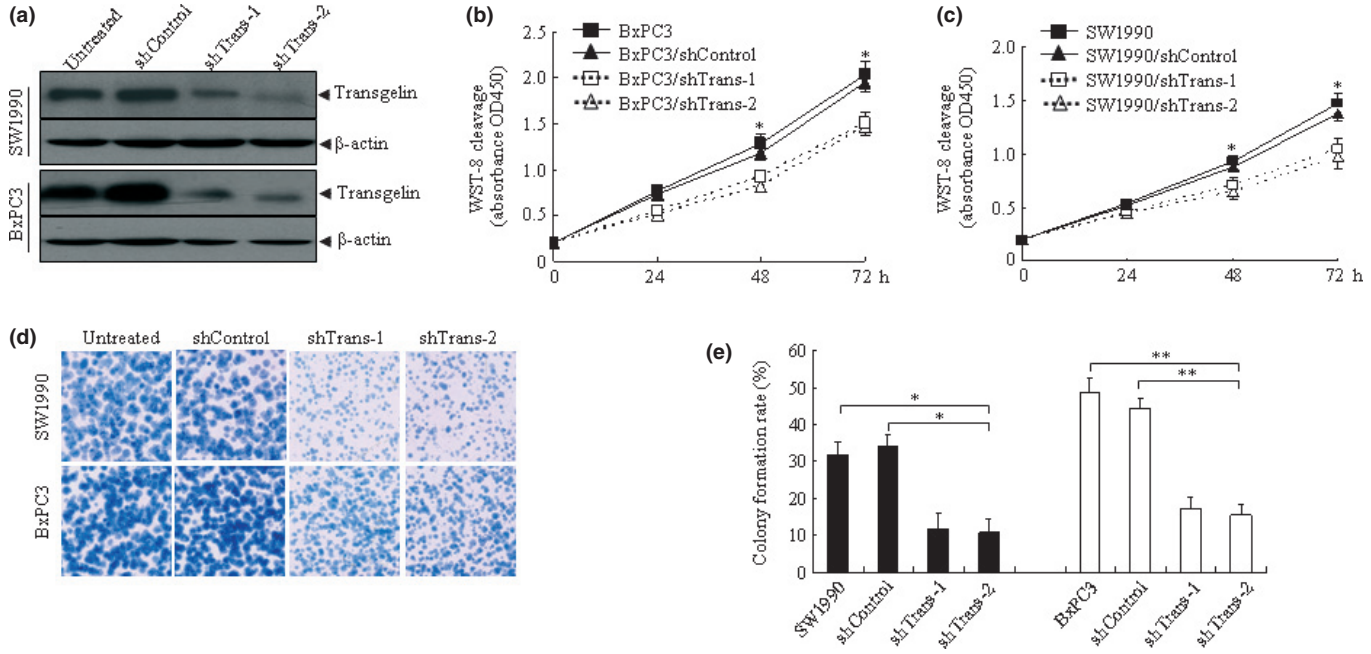

Fig. 3. Effects of transgelin on the growth and tumorigenesis of pancreatic cancer cells *in vitro*. (a) The expression of transgelin in SW1990 and BxPC3 cells stably transfected with pGCsi-H1-Transgelin plasmid (SW1990/shTrans-1, SW1990/shTrans-2 and BxPC3/shTrans-1, BxPC3/shTrans-2) were detected by western blot analysis. Untreated cells and cells transfected with pGCsi-H1/negative plasmid (SW1990/shControl, BxPC3/shControl) served as the controls. (b) BxPC3/shTransgelin, SW1990/shTransgelin cells and their controls were cultured for 24, 48 and 72 h. WST-8 cleavage in each group was measured (absorbance OD450). (c) Colony formation assay was performed using SW1990 and BxPC3 cells stably transfected with shTransgelin and shControl for 14 days. (d) Colony formation rates were analyzed by soft agar assay. All experiments were repeated in triplicate with similar results. $*P < 0.05$ and $**P < 0.01$ when compared to controls.

(shTrans-1) from each cell line for our subsequent *in vivo* studies and all results discussed hereafter are based on this clone. After the same amount of three types of cells was injected into mice, tumors appeared in all of the animals from SW1990 and SW1990/shControl groups at day 5. Mice that received injection of the SW1990/shTransgelin transfectants developed a relatively small tumor at a later time (day 8). There was a dramatic decrease in tumor volume and tumor weight in the SW1990/shTransgelin implantation group, as compared to the SW1990 and SW1990/shControl injection groups (Fig. 5a,b). The inhibitory effects of shTransgelin treatment and GEM were of similar magnitude. The co-treatment with shTransgelin and GEM showed a significant tumor growth inhibition as compared with shTransgelin + PBS and shControl + GEM groups. These results demonstrated that depletion of transgelin, in combination with GEM, significantly enhanced the anti-tumor effect *in vivo*.

Immunohistochemical staining showed that transgelin protein was expressed at a very low level in tumors derived from SW1990/shTransgelin transfectants but was highly expressed in tumors derived from SW1990/shControl transfectants and parental SW1990 cells (Fig. 5c). SW1990 and SW1990/shControl transfectants showed more and stronger tumor cell positive staining for PCNA protein than shTransgelin treatments. The combination group showed the faintest PCNA staining. These results suggested that transgelin could accelerate pancreatic cancer cell proliferation in nude mice.

Discussion

In the present study, we performed a patient-based immunohistochemical study to evaluate the expression level of transgelin in pancreatic cancer. Our findings are consistent with previous proteomics data that pancreatic cancer expresses a higher level of transgelin protein than normal pancreas. The upregulation of transgelin in pancreatic cancer may be due to the specificity of the hypoxic tumor-microenvironment. It is well known that pancreatic cancer is pathologically characterized as nests of neoplastic cells within an abundant fibrotic stroma, which leads to pancreatic cancer cells surviving and replicating within an extremely hypoxic environment.[22,23] Direct evidence that pancreatic cancer cells exist within a hypoxic environment has come from measurements of O_2 tension, which is significantly decreased in pancreatic cancer when compared with adjacent normal pancreas.[24] Investigators have shown that hypoxia could regulate transgelin expression both *in vivo* and *in vitro*. We previously showed that transgelin mRNA expression progressively rose in response to increasing periods of hypoxia in human pulmonary arterial smooth muscle cells.[25] Kim *et al.*[26] report that hypoxia-mediated induction of transgelin expression is hypoxia-inducible factor-independent in A549 non-small cell lung carcinoma cells (NSCLC). The same result was also observed in human breast epithelial cell line HBL100.[27] Therefore, the extremely hypoxic tumor-microenvironment might play a pivotal role in determining the upregulation of transgelin in pancreatic cancer cells.

The biological function of transgelin in cancers remains controversial. A major question that arises from this study appears to be whether transgelin overexpression in pancreatic cancer is a good or bad occurrence. Here, we demonstrate a significant correlation between transgelin expression and lymph node metastasis, which is a key factor in patient management and prognosis. We further found that patients with higher transgelin expression had unfavorable outcomes. As well as tumor grading and lymph node involvement, high expression of transgelin significantly correlated with poor tumor-specific survival and was an independent prognostic indicator for pancreatic cancer. These data suggest that transgelin acts as a tumor

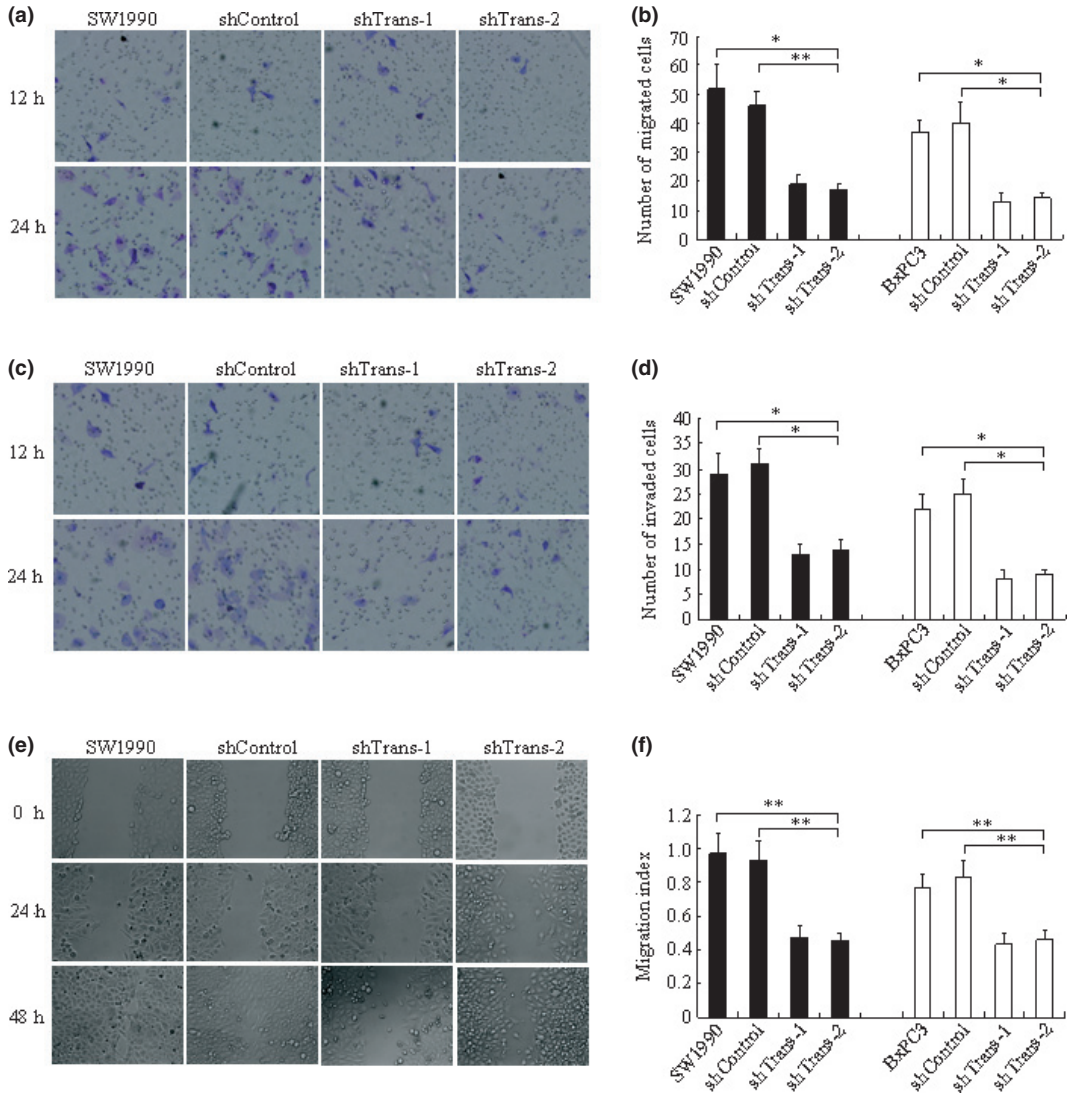

Fig. 4. Effects of Transgelin on the migration and invasion of pancreatic cancer cells. SW1990 and BxPC3 cells stably transfected with pGCsi-H1-Transgelin (SW1990/shTrans-1, SW1990/shTrans-2 and BxPC3/shTrans-1, BxPC3/shTrans-2) or control plasmids (BxPC3/shControl, SW1990/shControl) were subjected to migration, invasion and wound healing assays. Number of cells that migrated or invaded to the lower membrane surface was counted after 12 and 24 h. (a) Cell migration assay was performed using transwell chambers. Representative images are shown for SW1990. (b) Effects of transgelin on cell motility of SW1990 and BxPC3 at 24 h. (c) Invasive potential of cells across Matrigel-coated transwell chambers. Representative images are shown for SW1990. (d) Effects of transgelin on cell invasion of SW1990 and BxPC3 at 24 h. (e) Representative images at 0, 24 and 48 h after wound initiation are shown for SW1990. (f) Migration index was assessed by measuring the distance between the wound edges for SW1990 and BxPC3 at 48 h. All experiments were repeated in triplicate with similar results. $*P < 0.05$ and $**P < 0.01$ versus controls.

promoter and may contribute to the development of pancreatic cancer. Recent analysis has demonstrated the roles of transgelin in some types of cancer. Our findings are consistent with reports on the behavior of colorectal cancer[15] and gastric cancer,[14,28] although not with findings on esophageal cancer[11] and breast cancer.[29] The discrepancies between these results and our present data are not altogether surprising. The function of transgelin in cancer is reported to be dependent on cancer type and stage. Studies have identified that transgelin expression is under the control of the transforming growth factor β (TGF-β) signaling pathway in other systems.[30–35] TGF-β plays a paradoxical role in regulating tumor cell growth and migration.[36] During early tumor development, TGF-β functions as a tumor suppressor,[37] but in advanced pancreatic cancer, it switches to become a promoter of growth and invasion.[38] It may explain why expression is associated with tumor suppression in some studies and is a variable tumor biomarker in others. Notably, downregulation of transgelin has been reported in pancreatic intraepithelial neoplasia 2 (PanIN-2) grade, which is the precursor lesion of pancreatic ductal adenocarcinoma.[39] As most pancreatic cancer patients are diagnosed at an advanced stage, characterized by metastasis at the time of diagnosis, the contribution of transgelin in pancreatic cancer cannot be measured only by examining associations in patients with advanced pancreatic cancer. Further experiments, ideally using animal models for precancerous lesions, are merited to investigate the exact role of transgelin in pancreatic cancer.

In the present study, the upregulation of transgelin found in malignant ductal cells from patients with diabetes is noteworthy. Diabetes is now generally accepted as a crucial event in the process of pancreatic cancer. A recent population-based study shows that diabetes has a high (40%) prevalence in pancreatic cancer and frequently is new onset.[40] At least two possible

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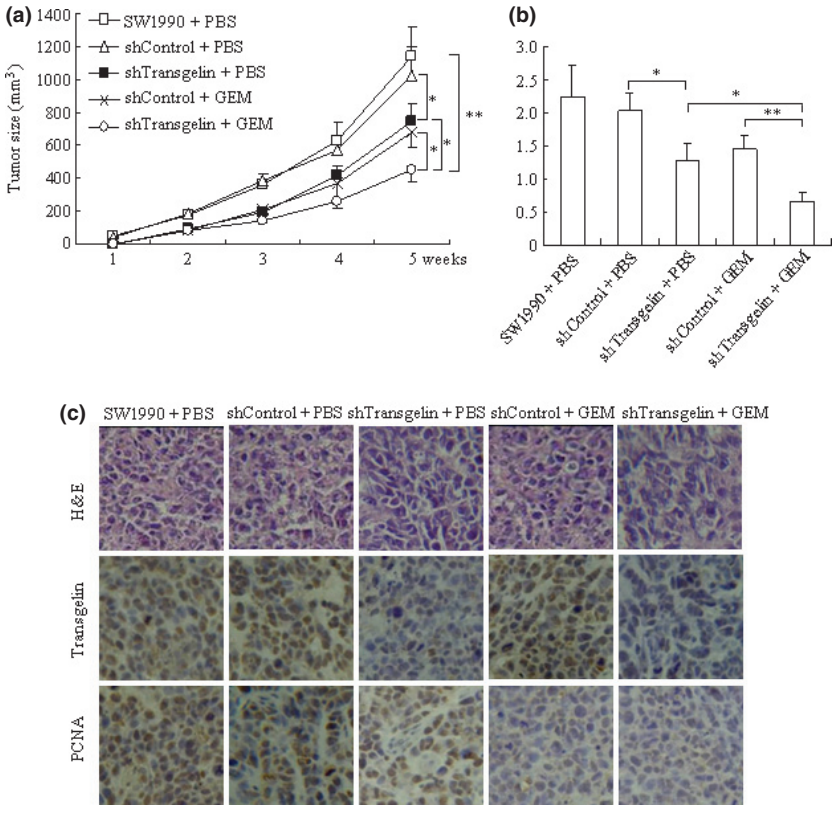

Fig. 5. Effects of Transgelin on tumorigenesis and tumor growth *in vivo*. Xenografted pancreatic cancers were established by s.c. injection of SW1990, SW1990/shControl and SW1990/shTransgelin (5×10^6) into 4-week-old Balb/c nude mice. Seven days later, they were administered either PBS or gemcitabine (GEM, 50 mg/kg) i.p., twice a week, respectively. (a) Tumor size was measured weekly. (b) Tumor weights were measured at day 35. (c) Immunohistochemical analysis of tumor sections from each treatment group, with antibodies against transgelin and the proliferation marker proliferating cell nuclear antigen (PCNA). *$P < 0.05$ and **$P < 0.01$ versus controls.

mechanisms might explain the relationship between transgelin expression and diabetic pancreatic cancer. First, diabetic patients show a close correlation with strong and longstanding inflammation induced by hyperglycemia. The expression of TGF-β, interleukin-6 and interferon-γ was found to be prominently increased in diabetic tissue.[41] Although we did not measure the levels of TGF-β in this study, it appears reasonable to speculate that the inflamed condition may consequently evoke transgelin protein expression via proinflammatory cytokines such as TGF-β. Next, higher levels of hypoxia in diabetic pancreas compared with non-diabetic pancreas is also, in part, responsible for the upregulation of the transgelin in malignant ductal cells.[42] It is conceivable that diabetic pancreatic cancer cells may exhibit a higher basal expression of transgelin compared with malignant cells in pancreatic cancer samples without diabetes.

In *in vitro* studies, our RNA knockdown experiments showed that two pancreatic cancer cell lines examined were sensitive to transgelin depletion, showing substantial reductions in cell proliferation and tumorigenicity. The motility data for transgelin are suggestive of potential roles for the protein in pancreatic cancer cell motility and consequently dissemination. In *in vivo* systems, depletion of transgelin expression in pancreatic cancer cells significantly suppressed the tumorigenicity and tumor growth in nude mice. Pancreatic cancer is known for its high chemotherapeutic resistance and poor treatment outcomes. We further compared the effect of transgelin-shRNA with that of GEM, an anticancer agent emerging recently as the first-line treatment of locally advanced and metastatic pancreatic cancers.[43,44] Suppression of transgelin expression enhanced the sensitivity of pancreatic cancer cells to GEM both *in vitro* (Document S2, Fig. S2) and *in vivo*. In A549 NSCLC cells, transgelin-overexpressing cells exhibit dramatic resistance to anticancer drugs and gamma-radiation. The mechanism seems to be that transgelin overexpression enhances tumor cell growth and activates the IGF1R/PI3K/Akt pathway through direct interaction with IGF1Rb.[26] These results provide preclinical evidence that pancreatic cancer expressing high levels of transgelin might be resistant to treatment, leading to a poor prognosis. Transgelin is a potential therapeutic target for treating pancreatic cancer.

Although the present study has focused on the proliferation and motility-associated function of transgelin in pancreatic cancer, it is entirely possible that the protein has other distinct roles in this disease. Kim *et al.* report that transgelin promotes migration and invasion of cancer stem cells, which possess the ability to initiate malignancies, promote tumor formation, drive metastasis and evade conventional chemotherapies.[45] More detailed molecular mechanisms of transgelin in pancreatic cancer need to be investigated.

In summary, we have demonstrated that transgelin is overexpressed in malignant pancreatic ducts and that such expression is an independent prognostic factor in patients with pancreatic cancer. Moreover, depletion of transgelin could significantly inhibit pancreatic cancer cell growth, motility and *in vivo* tumorigenicity. These results suggest that the actin-binding protein plays a promoting role in the development of pancreatic cancer. Further study into the role of transgelin in the precancerous pancreatic lesions–carcinoma sequence is merited.

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Disclosure Statement

The authors have no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Document S1. Methods of in vitro cell migration, invasion and wound-healing assays.

Document S2. Suppression of transgelin expression enhances the sensitivity of pancreatic cancer cells to GEM *in vitro*.

Fig. S1. Effects of siRNA on transgelin protein expression were confirmed by western blot.

Fig. S2. Effects of transgelin on the sensitivity of pancreatic cancer cells to GEM *in vitro*.

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