Signal Transduction

Stem Cell Marker Nestin Is Critical for TGF-β1-Mediated Tumor Progression in Pancreatic Cancer

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Abstract

The stem cell marker nestin is an intermediate filament protein that plays an important role in cell integrity, migration, and differentiation. Nestin expression occurs in approximately one third of pancreatic ductal adenocarcinoma (PDAC), and its expression strongly correlates with tumor staging and metastasis. Little is known about the mechanisms by which nestin influences PDAC progression. Here, nestin overexpression in PDAC cells increased cell motility and drove phenotypic changes associated with the epithelial-mesenchymal transition (EMT) in vitro; conversely, knockdown of endogenous nestin expression reduced the migration rate and reverted cells to a more epithelial phenotype. Mouse xenograft studies showed that knockdown of nestin significantly reduced tumor incidence and volume. Nestin protein expression was associated with Smad4 status in PDAC cells; hence, nestin expression might be regulated by the TGF-β1/Smad4 pathway in PDAC. We examined nestin expression after TGF-β1 treatment in human pancreatic cancer PANC-1 and PANC-1 shSmad4 cells. The TGF-β1/Smad4 pathway induced nestin protein expression in PDAC cells in a Smad4-dependent manner. Moreover, increased nestin expression caused a positive feedback regulator of the TGF-β1 signaling system. In addition, hypoxia was shown to induce nestin expression in PDAC cells, and the hypoxia-induced expression of nestin is mediated by the TGF-β1/Smad4 pathway. Finally, the antimicrotubule inhibitors, cytochalasin D and withaferin A, exhibited anti-nestin activity; these inhibitors might be potential antitumor drugs. Our findings uncovered a novel role of nestin in regulating TGF-β1-induced EMT. Anti-nestin therapeutics may serve as a potential treatment for PDAC metastasis. Mol Cancer Res; 11(7); 1–12. ©2013 AACR.

Introduction

The incidence of pancreatic ductal adenocarcinoma (PDAC) is rising to the fifth leading cause of cancer-related mortality. The condition is associated with the poorest prognosis among all gastrointestinal cancers, with a 5-year survival rate less than 4% (1, 2). A main reason for this extremely poor prognosis is the cancer’s tendency to invade adjacent tissues and metastasize to regional lymph at a relatively early stage. Patients with PDAC frequently experience disease recurrence with a high incidence of hepatic metastases, peritoneal dissemination, and lymph node as they undergo resection (3). These features underscore the pressing need to develop new therapeutic strategies specifically aimed at preventing invasion and metastasis in PDAC, to improve the survival rates and clinical outcomes.

Tumor metastasis is a complex phenomenon involving the extension of cell processes through the formation and/or disassembly of focal adhesion complexes and polymerized actin filaments (4, 5). Epithelial–mesenchymal transition (EMT) is a fundamental cellular process necessary for tumor metastasis (6, 7). EMT can be induced by a wide variety of stimuli, such as inflammatory cytokines [including interleukin (IL)-1, TNF-α, and TGF-β] or hypoxia (8–10). TGF-β1 has been described as a major EMT-inducing soluble factor in a range of tumors; this pleiotropic cytokine mediates the transcriptional regulation of downstream EMT-related genes through the formation of the Smad2/3–Smad4 complex (11–14). During PDAC carcinogenesis, TGF-β1 signaling is constitutively associated with tumor progression in advanced PDAC (15, 16). When treated with TGF-β1, many pancreatic carcinoma cell lines show a morphologic alteration in EMT, losing epithelial markers...
and gaining mesenchymal ones. This result confirms that TGF-β is one of the most prominent EMT inducers (13, 17, 18).

Tumor cells undergo EMT by losing epithelial characteristics such as cell adhesion, cell–cell contact, and remodeling of the cytoskeleton; simultaneously, they acquire mesenchymal phenotypes that facilitate invasion and migration to distal organs (19, 20). The cytoskeleton organizes other constituents of the cell, maintains cell shape, and is responsible for the internal movement of cell organelles and cell locomotion (21). Three major types of filaments comprise the cytoskeleton: actin filaments, microtubules, and intermediate filaments, which are known to be crucial modulators of the structural organization of cells and cell migration (22). Nestin is a large intermediate filament protein of class VI; it displays a short N-terminus and an unusually long C-terminus, which interacts with other cellular components, microtubules, and other intermediate filaments (including vimentin or desmin) to form heterodimers and mixed polymers. The long carboxy-terminal portion of nestin protrudes from the filament body and might function as a link or cross-bridge between the intermediate filaments and microtubule (23, 24).

Nestin was first described as a functional stem cell marker in embryonic and adult central nervous system (CNS) stem cells and is used extensively to identify progenitor cells in several tissues (25). In addition, recent research has characterized nestin as a cancer stem cell marker in various cancers; these include brain tumors, ovarian, head and neck, prostate, and pancreatic cancers (26–30). Nestin has also been implicated in pancreas development and pancreatic cancer progression (30). One in vitro study reported that nestin-positive cells derived from isolated pancreatic islets were able to differentiate into pancreatic endocrine or exocrine cells (31, 32). Moreover, Kawamoto and colleagues suggested that nestin expression in pancreatic cancer cells might be associated with nerve and stromal invasion (30). Matsuda and colleagues reported that nestin played an important role in pancreatic cancer cell migration, invasion, and metastasis by selectively modulating the expression of actin and cell adhesion molecules (33). Furthermore, in a previous study, nestin-expressing cells were reported to be the cells of origin for pancreatic intraepithelial neoplasia (PanIN) lesions (34). However, before our research, no study had attempted to determine the underlying mechanisms by which nestin promotes metastasis in pancreatic cancer.

In this study, we first determined that nestin expression may correlate with Smad4 status in PDAC. Furthermore, we found that nestin expression was necessary to mediate EMT induced by TGF-β1 and to promote PDAC migration and invasion. More important, nestin promoted TGF-β1/Smad signaling by increasing the expression of TGF-β1, TβR1, and TβR2, which led to constitutively active TGF-β1/Smad signaling in nestin-positive PDAC cells. These results provided a molecular-level understanding of nestin action in pancreatic cancer metastasis.

Material and Methods

Cell culture, RNA isolation, and cDNA synthesis
Immortalized normal human pancreatic ductal cells (HPDEC) were obtained from Dr. Tsao (Ontario Cancer Institute, Princess Margaret Hospital, University Health Network, Toronto, Ontario, Canada) (35). The HEK293T and human PDAC cell lines were obtained from the sources described previously (36, 37). We administered TGF-β1 (5 ng/mL), hypoxia, and inhibitors treatment according to previously described procedures (37). The RNA isolation and cDNA synthesis from the cell lines were also conducted according to previously described procedures (38, 39).

Lentivirus production and shRNA for gene knockdown
The plasmids required for short hairpin RNA (shRNA) lentivirus production were purchased from the National RNAi Core Facility, Academia Sinica (Taipei, Taiwan). The pLKO.1-shRNA vectors used for knockdown of nestin and smad4 were TRCN0000014729 (Nestin) and TRCN0000010323 (Smad4). The pLKO.1-shEGFP control plasmid was TRCN00000-72190 (EGFP). Lentivirus production and infection were conducted according to a previously described protocol (39).

Western blotting and immunofluorescence
Western blotting and immunofluorescence analyses were conducted as previously described (36, 39). Primary antibodies used were nestin antibodies (Sigma); smooth muscle actin (SMA, ab5698); N-cadherin (ab12221); TGF-β1 (ab66043; Abcam); E-cadherin (sc-8426); vimentin (sc-32322); Smad4 (sc-7966); CD44 (sc-18849; Santa Cruz Biotechnology); and mouse anti-B-actin (Sigma). E-cadherin (#4065), Smad2 (#3122) and p-Smad2 (#3104; Cell Signaling).

Quantitative RT-PCR analysis
Quantitative real-time PCR (qRT-PCR) amplification and results of the ∆Ct measurements were conducted as described in previously published methods (39). The primer sequences were listed in Supplementary Table S1.

Transient transfections and luciferase reporter assays
EGFP-C1-nestin expression vector and pNesPE-Luc (with 5’ flanking 4 kb promoter + intron II) reporter construct were provided as a gift from Dr. Jing [Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences, Shanghai, China]. Transient transfections and luciferase reporter assays were conducted as previously described (39–41).

In vitro migration/invasion assay
For Transwell migration and the wound closure assays, cells were pretreated with 0.02% (0.2 mg/mL) mitomycin C and followed previously published protocols (37, 39).

In vivo xenograft mouse study
Specific pathogen-free male C.B17/crl-SCID mice, 8 weeks of age, were purchased from BioLASCO Taiwan Co., Ltd.. Mice were treated according to the institutional
guidelines for the care and use of experimental animals. The detailed protocol for establishing subcutaneous injection of xenograft tumor models in severe combined immunodeficient (SCID) mice was conducted using our previously described method (39), and orthotopically grown pancreatic cancer xenografts were conducted according to a previously publication (42).

Mice strains for genetically engineered mouse models of PDAC, necropsy, histopathology, and immunohistochemistry

The mouse strains included in this study were PDX-Cre and LSL-Kras<sup>G12D</sup>, which were obtained from National Cancer Institute Mouse Models of Human Cancers Consortium (NCI-MMHCC). All experiments were carried out on a more than 96.9% C57BL/6J background. Mice genotyping, tissue processing, and IHC staining were conducted as previously described (36, 43). The IHC analysis was conducted as previously described using anti-Smad4 (Santa Cruz Biotechnology), anti-vimentin (Santa Cruz Biotechnology), anti-nestin (Sigma), anti-nestin antibody (clone 10C2; Abcam), anti-E-cadherin (Santa Cruz Biotechnology), anti-CD44 (Santa Cruz Biotechnology), and anti-TGF-β (Santa Cruz Biotechnology). Images were captured using a Carl Zeiss. Axioskop 2 plus microscope (refs. 36, 44).

Human samples

Human PDAC tumor tissues were collected during surgical resections at Chung-Ho Memorial Hospital of Kaohsiung Medical University (KMU; Kaohsiung, Taiwan). The surgically resected tumor samples were immediately fixed in 10% formalin and embedded in paraffin (FFPE) using standard protocols (39). Sections from each specimen were confirmed by pathologists and graded histologically. Representative FFPE human PDAC blocks were further selected for analysis of nestin. The study protocol was approved by the Institutional Review Board at KMU (39).

Statistical analysis

Data are presented as mean ± SEM. The data measured on continuous scale were statistically analyzed using Student t test and categorical data were subjected to χ<sup>2</sup> test. A P value of less than 0.05 was considered significant.

Results

Nestin expression in the normal versus malignant pancreas

To study the role of nestin in the pancreas and malignant formation, we analyzed nestin expression in the pancreas using IHC and immunofluorescence analyses. Consistent with some previous reports, our findings revealed that nestin-positive cells were observed mainly in the cytoplasm of a limited number of vascular endothelial cells, intrapancreatic nerves, and within the islets of normal (mouse and human) pancreatic tissues. Weak or no detectable expression of nestin was evident in the pancreatic acinar cells (Fig. 1A and B; Supplementary Fig. S1; refs. 44, 45). We also stained tissues from a set of mouse and human malignancies. In the PanIN lesions of Pdx-Cre LSL-KrasG12D mice, IHC and immunofluorescence analyses indicated an increase in nestin protein expression in the cytoplasm. PanIN lesions in Pdx-Cre KrasG12D mice showed strong positive staining for nestin, which implied that nestin expression was induced...
from an early stage of pancreatic malignant transformation (Fig. 1A).

To verify this finding, we further conducted IHC and immunofluorescence analyses to evaluate the nestin expression in human PDAC tissues. Nestin expression was observed mainly in the cytoplasmic region of malignant ductal structures; the same pattern has been described for mouse PDAC tissues (Fig. 1A and B). Next, we further evaluated nestin expression in 6 human PDAC cell lines by Western blot analysis. Nestin was expressed in various amounts in the pancreatic cancer cell lines tested, with the highest expression observed in wild-type Smad4-expressing HPDEC, PANC-1, and MiaPaCa-2 cells; relatively low expression was observed in Smad4-mutated or null PDAC cells (AsPC-1, BxPC3, and CFPAC-1). As shown in Fig. 1C, nestin was highly expressed in Smad4-positive PDAC cells. Our findings indicated that signaling through Smad4 may regulate nestin expression in PDAC cells. Because Smad4 is a central signaling molecule of TGF-β—induced signal transduction, we hypothesized that nestin expression status might depend on TGF-β1/Smad4 signaling.

**Nestin promoted tumor cell migration and induced EMT in PDAC**

We investigated whether nestin played a direct functional role in facilitating PDAC cell migration. Three independent PDAC cell lines, namely PANC-1, AsPC-1, and MiaPaCa-2, were selected to examine the expression and role of nestin in regulating PDAC cell migration. We induced the overexpression of nestin in MiaPaCa-2 or AsPC-1 cells by transfecting the cells with the EGFP-C1-nestin expression vector to overexpress nestin protein (see Materials and Methods). Nestin knockdown was conducted using nestin lentiviral shRNA in PANC-1 cells, and stable cell lines resistant to puromycin were selected. Cell migration and invasion were further evaluated by using the wound-scratch method and SPL insert Transwell system using a track-etched polycarbonate membrane, pretreated by coating with type I collagen (2 mg/mL). As shown in Fig. 2A, the PANC-1 nestin shRNA cells displayed significantly lower closure compared with the control cells ($P < 0.05$). We then extended our experiments to examine the overexpression of nestin in other PDAC cell lines (AsPC-1, MiaPaCa-2, and BxPC3). The results confirmed that nestin promoted PDAC cell migration.

Next, *in vitro* invasion was measured by the number of tumor cells that penetrated the collagen-coated membrane of the insert and attached themselves to the bottom well. Invasion was further assessed by counting the number of cell colonies under a microscope after crystal violet staining. Our findings showed that the invasion of PANC-1 nestin shRNA cells was attenuated compared with the control (Fig. 2B). Similar results were obtained from nestin-overexpressing MiaPaCa-2 cells compared with vector control cells; these findings confirmed that overexpression of nestin increased PDAC cell invasive potential *in vitro*.

The EMT process is necessary for cell migration; therefore, we attempted to uncover the molecular mechanisms involved in regulating nestin-activated PDAC cell migration. We examined the effects of nestin expression in the regulation of EMT. First, we used qRT-PCR analysis to examine EMT-related markers in PANC-1 nestin shRNA cells and GFP control cells; the examined markers included mesenchymal markers of vimentin, SMA, and N-cadherin, epithelial marker of E-cadherin, and nestin mRNA levels. The results of our qPCR analysis showed that knockdown of nestin increased the expression of E-cadherin mRNA and decreased the levels of vimentin, SMA, and N-cadherin mRNA (Fig. 2C). Similar results were obtained using nestin-overexpressing MiaPaCa-2 cells compared with control. Western blotting was conducted to detect the protein expressions of EMT-related markers in PANC1 nestin shRNA cells and nestin-overexpressing MiaPaca-2 cells versus controls. The Western blot analysis results were similar to the preceding analysis; protein levels of vimentin, SMA, and N-cadherin had decreased significantly and E-cadherin protein had increased in PANC-1 nestin shRNA knockdown cells compared with the control cells. Similar results were obtained using nestin-overexpressing MiaPaCa-2 and AsPC-1 cells, compared with controls (Fig. 2D).

**Nestin expression is induced by TGF-β1–Smad4 pathway**

Our previous findings confirmed that nestin is involved in the EMT process. The TGF-β1–Smad signal is known to be a potent inducer of EMT during carcinogenesis. Nestin expression may be associated with Smad4 status in human PDAC cells. To verify the interactions between nestin and TGF-β/Smad signaling, we selected PANC-1 (Smad4-positive) and AsPC-1 (Smad4 null) PDAC cell lines for further study. Our findings indicated that the TGF-β/Smad pathway induced nestin expression, downregulated E-cadherin, and increased protein expression of vimentin, SMA, and N-cadherin (Fig. 3A). Most important, the TGF-β pathway was found to induce nestin protein expression, and this induction was mediated by Smad4-dependent activation (Fig. 3B and C). Furthermore, administration of SB525334 (20 μmol/L; Sigma-Aldrich) inhibited the TGF-β pathway in PANC-1 cells, and led to a significant reduction in nestin expression, as shown in the Western blot analysis (Supplementary Fig. S2).

To confirm these findings, we used the nestin reporter construct pNesPE-Luc to verify that nestin transcriptional activity was increased by the TGF-β1/Smad4 pathway in PANC-1 Smad4-positive cells to confirm that Smad4 was necessary for TGF-β–induced nestin expression, and 3TP-TGF-β1 reporter construct was used to determine the active TGF-β1/Smad pathway (Fig. 3D). Rapid tumor growth frequently led to hypoxia, which in turn influenced signaling events within the tumor cell and eventually activated an angiogenic program and EMT. Previous studies have shown that the TGF-β pathway can be
induced by hypoxia, which can further drive TGF-β-induced EMT by several pathways. To examine whether hypoxia stimulated TGF-β1 expression in PDAC cells, we incubated cells under normoxic (N) or hypoxic (2% O2) conditions to detect TGF-β1 protein expression. Under the hypoxic condition, we found a marked increase in TGF-β1 protein expression and an increased expression of nestin in PANC-1 cells (Fig. 3E). We also found that hypoxia-inducible factor-1 inhibitor, LW6 (10 μmol/L; Merck Millipore) reduced the expression of nestin in PANC-1 cells by qRT-PCR analysis (Supplementary Fig. S3). We speculated that hypoxia induced an increase in the expression of TGF-β1 cytokine, leading to the induction of nestin expression under hypoxic conditions. Hypoxia-induced TGF-β1 protein expression was abolished after knockdown of Smad4 or nestin expression in PANC-1 cells (Fig. 3F).

Nestin is involved in TGF-β1/Smad4 signaling to induce EMT in PDAC

To test if nestin was involved in TGF-β–induced cell migration, we examined the function of nestin in TGF/Smad signaling. Western blot analyses were conducted to detect the phosphorylation levels of Smad2 in PANC-1 nestin shRNA clonal line and control. Western blot analyses were also conducted in nestin-overexpressing BxPC-3, AsPC-1 (b) and MiaPaCa-2 cells, or in an shRNA eGFP versus PANC-1 nestin shRNA stable transfected cells (a). Total lysates were analyzed for protein levels of nestin, E-cadherin, N-cadherin, vimentin, SMA, and β-actin.
To explore the mechanism by which nestin expression promotes the elevation of p-Smad2 levels, we determined the expression level of TGF-β cytokine by Western blot analysis and qRT-PCR analysis. Our findings indicated that knockdown of nestin expression in PANC-1 cells resulted in reduced TGF-β1 expression at the mRNA and the corresponding protein levels (Fig. 4B and D). Consistent with this finding, we also detected an increase in TGF-β1 protein expression in nestin-overexpressing MiaPaCa-2 (Smad4-proficient) and AsPC-1 (Smad4-deficient) cells by Western blot analysis (Fig. 4B and Supplementary Fig. S4). Furthermore, we investigated whether TGF-β activity was altered after knockdown or overexpression of nestin. Luciferase reporter assays with various TGF-β1-Smad signaling responsive reporter constructs were used to confirm that nestin modulated the activation of the TGF-β1-Smad signaling pathway in PANC-1 cells (Fig. 4C). Our findings indicated that nestin knockdown resulted in the downregulation of the TGF-β1-Smad pathway. A similar result was observed in an increase of the TGF-β1/Smad-responsive reporter 3TP-luciferase (3TP-Luc) was used to confirm TGF-β1 treatment. E, total lysate proteins isolated from MiaPaCa-2 and PANC-1 cells under normoxia and hypoxia treatments were subjected to Western blotting for nestin, vimentin, TGF-β1, and β-actin. F, immunoblot analysis of TGF-β1 from PANC-1 nestin shRNA and Smad4 shRNA cells compared with control eGFP shRNA cells.

Previous studies have indicated that TGF-β interacts with the Wnt pathway to modulate EMT at various stages of development. Our findings showed a significant reduction in transcription activity of the Wnt transcriptional reporter Super TOPFlash and Wnt downstream gene CD44 reporter in PANC-1 nestin shRNA cells, compared with controls (Fig. 5A), which may explain why we next examined the consequence of knockdown of nestin expression on the regulation of EMT markers expression after TGF-β1 treatment. Western blotting revealed that the protein level of the mesenchymal marker vimentin was significantly diminished and the epithelial marker of E-cadherin expression was high in PANC-1 nestin shRNA cells, irrespective of whether they were treated with TGF-β1 overnight (Fig. 5B). This may explain why we observed that PANC-1 nestin shRNA cells grow in piled-up structure of irregular round cells (Fig. 5C). In contrast, TGF-β1 treatment of mock-transfected

Figure 3. TGF-β1–Smad4 signaling regulated nestin expression. A, immunoblot analysis of nestin, TGF-β1 pathway, and EMT-related proteins expression in indicated cell lines. B, Western blot analysis of nestin and vimentin expression in PANC-1 cells after TGF-β1 treatment. C, representative Western blot analysis depicting the elevation of nestin expression in Smad4-positive PANC-1 cells treated with or without TGF-β1. D, Western blot analysis of nestin and vimentin expression in PANC-1 cells after TGF-β1 treatment, compared with PANC-1 smad4 shRNA cells treated with or without TGF-β1. The TGF-β1–Smad–responsive reporter 3TP-luciferase (3TP-Luc) was used to confirm TGF-β1 treatment. E, total lysate proteins isolated from MiaPaCa-2 and PANC-1 cells under normoxia and hypoxia treatments were subjected to Western blotting for nestin, vimentin, TGF-β1, and β-actin. F, immunoblot analysis of TGF-β1 from PANC-1 nestin shRNA and Smad4 shRNA cells compared with control eGFP shRNA cells.
Nestin Mediates TGF-β1–Induced EMT and Tumor Progression

Figure 4. Nestin activates TGF-β1 signaling and induces an upregulation of TGF-β1 and its receptors expression in PDAC cells. A, Western blot analysis of total Smad2 and p-Smad2 protein levels in protein lysates isolated from the eGFP control and nestin shRNA-transfected PANC-1 cells (a), or in nestin-overexpressed MiaPaCa-2 cells and GFP control (b). B, Western blot analysis of TGF-β1 in nestin shRNA transfected PANC-1 cells (a) and nestin overexpressed MiaPaCa-2 cells (b) and their corresponding controls. C, activation of TGF-β1 signaling pathways was measured by different TGF-β1 responsive reporter constructs (3TP-Luc; SBE4-Luc and PAI-1-Luc) in nestin shRNA-transfected PANC-1 cells (a) and nestin-overexpressed MiaPaCa-2 cells (b) and their corresponding controls. Mean ± SEM (n = 3). *P < 0.05. D, qRT-PCR analysis of TGF-β1, TGF-βRI, TGF-βR2, and Smad7 mRNA levels from total RNA extracted from the eGFP control and nestin shRNA-transfected PANC-1 cells (a), or in nestin-overexpressed MiaPaCa-2 cells and GFP control (b). Results show the mean of 3 experiments ± SEM and are normalized to the GAPDH gene.

Figure 5. Nestin knockdown reduces in vivo tumorigenicity and inhibited TGF-β1/Smad4–mediated EMT in PANC-1 xenograft tumors

Nestin knockdown reduces in vivo tumorigenicity and inhibited TGF-β1/Smad4–mediated EMT in PANC-1 xenograft tumors

Nestin has been associated with mitotic spindle organization and mitosis, and cytokinesis plays a key role in cell proliferation (46). We thus investigated the function of nestin in PDAC tumorigenesis in vivo. We subcutaneously injected the left and right flanks of SCID mice with stable PANC-1 transfected with nestin shRNA or EGFP shRNA control cells, to confirm the in vivo effect of nestin on pancreatic carcinogenesis. After 2 months, mice that had been injected with the EGFP control or nestin shRNA-transfected cells showed the expected tumor formation. We conclude that PANC-1 nestin shRNA cells display reduced tumorigenic properties in vivo, compared with the control groups. Tumor sizes and weights were also 3- to 5-fold smaller than in controls (Fig. 6A). Hematoxylin and eosin (H&E) staining and IHC assays were conducted to examine the xenografts in the SCID mice generated from the injected PANC-1 cells (nestin shRNA vs. EGFP shRNA control; Fig. 6B).

Histopathologic analyses of representative tumor sections showed that the wild-type PANC-1 tumors generally comprised spindle-shaped cells with a slender cytoplasm, often with elongated cytoplasmic processes. In contrast, PANC-1 nestin shRNA tumor cells appeared less dense, with a more flattened morphology resembling cobblestones. Analysis of nestin expression by immunohistochemistry showed a reduced cytoplasmic expression in PANC-1 nestin shRNA tumors. In these tumors, E-cadherin staining was modestly increased but the expression of vimentin decreased, whereas it was high in the wild-type controls (Fig. 6B). Moreover, CD44 staining resulted in decreased protein staining of the membrane and cytosol when nestin was knocked down. We also observed that nestin shRNA PANC-1 tumors displayed decreased TGF-β1 expression and an inhibition of TGF-β1/Smad signaling, with greater Smad4 staining observed in the cytoplasm, compared with the control groups (Fig. 6B).
Identification of antinestin drugs with in vitro and in vivo antimetastatic activity

We selected several potential intermediate filament–targeted inhibitors to test if these compounds might display antinestin effects or anti-invasive (antimetastatic) properties in PDAC. We selected 4 antitumor drugs, namely cisplatin (a DNA-damaging agent), paclitaxel (an antimitotube compound), cytochalasin D (antiactin agent), and withaferin A (an antimetastatic agent; refs. 47, 48). For the cytotoxicology experiments, we first determined the \( IC_{50} \) values of each compound and applied a dose 5-fold below the \( IC_{50} \) value, to eliminate a possible cytotoxic effect on proliferation and to investigate the drug’s antinestin activity in vitro. The cytoxic drug cisplatin was used as a positive control for apoptotic analysis. To investigate the effect of the inhibitors on cellular nestin, cells were treated with 0.5 \( \mu \)mol/L cisplatin, paclitaxel, withaferin A, and cytochalasin D for overnight, and then cell extracts were analyzed by Western blotting. The results showed that both withaferin A and cytochalasin D treatment were able to inhibit the nestin protein level (Fig. 7A). This conclusion was also confirmed by immunofluorescence analysis (Fig. 7B).

Finally, we investigated whether withaferin A and cytochalasin D antinestin inhibitors displayed antinestin activity in vitro using in vitro Transwell migration assays. The invasion of PANC-1 cells treated with 0.5 \( \mu \)mol/L of cytochalasin D and withaferin A had decreased significantly compared with control or cisplatin groups \((P < 0.01)\). We used quantitative analysis to determine the number of PANC-1 cells treated with cytochalasin D or withaferin A that migrated to the lower side of the bottom wells using 0.5% crystal violet staining. The results indicated a 3-fold reduction in cell migration activity compared with the cisplatin or control groups (Fig. 7C). For in vivo metastasis analysis, we first used a commonly used Transwell cell migration assay to enrich high metastatic PANC-1 cells and repeated 6 times for enrichment of nestin\(^{\text{high}}\) subpopulation of PANC-1 cells (Supplementary Fig. S5A). After the final round of enrichment, migrated cells were observed with high-level expression of nestin as compared with the parental PANC-1 cell line by Western blot analysis (Supplementary Fig. S5B). PANC-1 nestin\(^{\text{high}}\) cells were then orthotopic injected in to the pancreas of SCID mice for in vivo metastasis analysis to determine the in vivo effects of cytochalasin D and withaferin A inhibitors on our orthotopic PDAC xenograft models. Our results confirmed the cytochalasin D or withaferin A treatment suppresses PDAC tumor development and metastasis formation in vivo (Supplementary Fig. S5C).
Discussion

Nestin, a class VI intermediate filament, similarly to other eukaryotic intermediate filaments, connects the 3 components of the cytoskeleton and coordinates changes in the cell dynamics (49, 50). Nestin reportedly interacts with vimentin or desmin to form heterodimers or polymers; these structures provide cellular mechanostructural support, maintain cellular membranes, and restrict organelles to a limited area (51). Nestin has also been found to contribute to the disassembly of vimentin during mitosis, to regulate the proapoptotic cyclin-dependent kinase 5, and to modulate mitosis-associated cytoplasmic reorganization after becoming phosphorylated on Thr316 by Cdc2 kinase (52). One recent study indicated that nestin may modulate actin and cell adhesion molecules to control pancreatic cancer cell migration, invasion, and metastasis (33). However, the signal mechanisms regulating nestin expression during migration remain unknown.

Cell migration is initiated or induced by activation of receptors that trigger remodeling of the cytoskeleton and reordering of subcellular organelles. EMT is increasingly recognized as a mechanism, whereby cells in primary noninvasive tumors acquire properties essential for migration and invasion (6, 53). The EMT process is regulated by the secretion of several factors in the tumor microenvironment, such as IL-6 or TGF-β. The TGF-β1 signaling is modulated by a number of biochemical interactions, including the association of Smad2/3 with various transcriptional cofactors to modulate Smads complex stability and possibly also to activate certain non-Smad pathways (8, 13). Studies using mutant TGF-βRII constructs that are defective in binding Smads but which retain signaling by mitogen-activated protein kinases have shown that Smad pathway is likely to be involved in the EMT process (54).

Our previous study on animal models of PDAC suggested that TGF-β1 signaling is activated in PanINs and advanced cancers; this finding was consistent with observations in human PDAC samples. On the basis of the findings from our previous research and the current study, we speculate that the excessive TGF-β1 cytokine observed around PanIN lesions results in increased nestin expression. Subsequently, under this late-stage tumor burden, hypoxia greatly increases TGF-β1 stimulation of nestin expression, which in turn facilitates tumor cell invasion and metastasis to distant sites. Our engineered mouse models suggested that active TGF-β1 is required for tumorigenicity of certain subsets of PDAC characterized by expression of wild-type Smad4 and to acquire a highly invasive and undifferentiated phenotype and more metastatic behavior (36). Moreover, the increased TGF-β1 observed in the blood of patients with PDAC is correlated with poor prognosis and survival, suggesting that TGF-β1 is an important marker for cancer progression (55).

In summary, our findings highlight the need for further functional studies on the role of nestin in TGF-β1-induced EMT and tumor metastasis. We confirmed that nestin overexpression induces the EMT of PDAC cells. We showed that TGF-β1 induced the expression of nestin in PDAC cells and that this induction was predominantly mediated by the
Smad4-dependent pathway. Overexpression of nestin was shown to increase the Smad2 phosphorylation level, leading us to hypothesize that nestin might function as a positive regulator of TGF-\(\beta_1\) signaling. This notion was supported by evidence that the overexpression of nestin induced TGF-\(\beta_1\) and the expression of its receptors at the RNA and protein levels through the Smad4-dependent pathway. Thus, nestin-positive cells apparently use an autocrine positive feedback loop to maintain the constitutive activation of TGF-\(\beta_1\) signaling in PDAC.

This process might play an important role in supporting the distant metastasis of PDAC in vivo. Three main factors would explain this phenomenon, namely (i) the overexpression of nestin maintains PDAC cells in a mesenchymal-like morphology with a high metastatic ability; (ii) nestin increases the cellular production of TGF-\(\beta\), which acts on surrounding cells to release further matrix metalloproteases and secrete VEGF; it also facilitates the degradation of extracellular matrix and promotes tumor progression; and (iii) excess production and/or activation of TGF-\(\beta_1\) by nestin-positive PDAC cells may mediate the suppression of immune responses by suppressing IL-2 production, inhibiting CTL activation, and inhibiting Toll-like receptor expression (56, 57); these factors help nestin-positive tumor cells escape host immune surveillance and gain a further advantage in growth.

This study reports novel findings of a positive cross-regulatory loop between nestin-TGF-\(\beta_1\)/Smad and EMT (Fig. 7D). This nestin–TGF-\(\beta_1\) relationship creates a positive feed-forward loop that controls PDAC tumor metastasis. Overall, our findings provide preliminary evidence and support the hypothesis that nestin might play a role in autologous tumor metastasis promotion by autologous activation of TGF-\(\beta_1\)/Smad signaling in PDAC. This hypothesis could explain how nestin-mediated signal pathways contribute to the cancer stem cell phenotype, and would imply that nestin is critical to maintain the

Figure 7. Antimicrotubule inhibitors withaferin A and cytochalasin D targeted nestin protein expression and inhibited cell invasion in PDAC cells. A, PANC-1 cells and MiaPaCa-2 cells under treatment with different inhibitors for 24 hours. Cell lysates were analyzed for nestin expression by Western blotting with a specific antibody. B, immunofluorescence analysis was conducted on PANC-1 cells to detect alterations in nestin proteins after 24-hour treatment with dimethyl sulfoxide (DMSO), cisplatin (Cis), withaferin A (WFA), and cytochalasin D (CD). Nuclei were visualized with 4’,6-diamidino-2-phenylindole (DAPI; blue). Images were taken at \(\times 400\) magnifications. C, in vitro Transwell invasion assays in PANC-1 and MiaPaCa-2 cells treated with cisplatin, withaferin A, or cytochalasin D, and their respective controls. Bar graphs show the relative invasive ability of PANC-1 and MiaPaCa-2 cells treated with DMSO, cisplatin, withaferin A, or cytochalasin D. The quantitative results were normalized in relation to DMSO control groups (mean \(\pm\) SD; \(n = 3\); \(P < 0.01\)). D, proposed molecular mechanism of nestin mediated TGF-\(\beta_1\)-induced EMT and plays positive feedback regulator of TGF-\(\beta_1\)/Smad signaling after hypoxic stimulus. Increased nestin expression led to enhanced TGF-\(\beta_1\) or its receptors expression to further support an autocrine TGF-\(\beta_1\) signaling cascade. Pac, paclitaxel.
progression of tumor metastasis. Although our study does not describe the entire molecular scenario for distant metastasis in PDAC, it nevertheless makes a major contribution to the theory of a metastatic PDAC network and outlines a strategy here for the treatment of invasion and metastasis by PDAC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.-C. Weng, P.-J. Hsiao, L.-H. Chen, T.-L. Kao, K.-K. Kuo, K.-H. Cheng
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Molecular Cancer Research

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Mol Cancer Res  Published OnlineFirst April 3, 2013.

Updated version  Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-12-0511

Supplementary Material  Access the most recent supplemental material at: http://mcr.aacrjournals.org/content/suppl/2013/04/02/1541-7786.MCR-12-0511.DC1

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