Introduction

The transition of epithelial cells to a mesenchymal phenotype (EMT) is a fundamental characteristic of carcinoma cells [1]. A lineage tracing study using genetically engineered mouse models of pancreatic adenocarcinoma demonstrated that EMT of pancreatic epithelial cells leads to their migration into surrounding stroma and entry into the bloodstream. Importantly, these events were observed before the formation of a solid tumor in the animals [2]. These data suggest that seeding of distant organs occurs before pancreas tumor formation, an observation whose clinical relevance is supported by the high rate of metastasis experienced by patients with pancreatic cancer [3]. In humans, pancreatic inflammation is strongly associated with the subsequent development of pancreatic cancer. The animal lineage tracing study found that inflammation in the form of pancreatitis increased EMT and subsequent dissemination into the bloodstream [2]. Therefore, observations in both mouse models and patients identify inflammation-related EMT of pancreatic epithelial cells as an outcome-determining event in pancreatic cancer.

A major constituent of this process is the interaction between the pleiotropic cytokine transforming growth factor-β (TGF-β) and cadherins, which are transmembrane glycoproteins that mediate calcium-dependent cell–cell adhesion. TGF-β, an abundantly studied inducer of EMT, has been shown to regulate tissue homeostasis and prevent tumorigenesis. TGF-β dimers bind to TGF-β type II receptors, which phosphorylate TGF-β type I receptors via serine/threonine kinase activity, which in turn phosphorylate cytoplasmic SMAD2 and SMAD3. The phosphorylated SMAD protein then binds to SMAD4, which is subsequently translocated into the nucleus. The complex then binds
gene promoter regions termed SMAD-binding elements (SBEs) in order to regulate transcription. Jonk et al reported the identification of SBEs composed of the sequence CAGACA in the promoter of the JunB gene, which is potently induced by TGF-β and the related cytokines activin and bone morphogenetic protein (BMP) [4]. Others also identified the 8-bp palindromic sequence GTCTAGAC as a SBE [5–7]. TGF-β signaling can also be transduced through a non-canonical pathway, such as the ERK, JNK, and MAPK pathways, as well as some small GTPase pathways [8,9].

SMAD4 is also considered a tumor suppressor gene that was originally recognized as “deleted in pancreatic carcinoma locus 4” (DPC4) on chromosome 18q21.1 [10,11]. As a tumor suppressor, SMAD4 has been extensively analyzed, but reports of its function in EMT have been contradictory. SMAD4/DPC4 protein functions are required in the regulation of TGF-β-inducible EMT, which plays an important role in embryogenesis, cell adhesion, cellular motility, and cancer cell invasion and metastasis [12–15].

One characteristic phenotypic change of EMT is the upregulation of N-cadherin. The gene that encodes for N-cadherin, CDH2, is a classic gene of the cadherin superfamily. This gene is expressed mainly in mesenchymal cell types, including nerve tissues, myocytes, and fibroblasts [16,17]. Expression of N-cadherin has been reported to enhance the invasive capacity, cell migration, metastasis, and angiogenesis of a variety of cancers, including those of the bladder, breast, esophagus, and thyroid [18–20]. Knockdown of N-cadherin in the BxPC-3 pancreatic cancer cell line was shown to lead to decreased tumor size and metastases in an orthotopic animal model [21]. These studies suggest that N-cadherin plays an important role in cancer metastasis and that understanding its regulation and function could help us to understand better of SMAD4/N-cadherin related cell motility, and may explain mechanism of pancreatic tumor metastasis.

As TGF-β induces EMT and the TGF-β signaling pathway is transduced by SMAD4, our study has focused on how the SMAD4 restrains N-cadherin expression in human pancreatic ductal epithelium, we hypothesize that SMAD4, through the binding of SBEs, regulates N-cadherin expression and cell invasion and migration.

**Materials and Methods**

**Ethics statement**

All patients in the study signed written informed consent prior to undergoing planned pancreatectomy. Each patient was given the opportunity to either consent or decline to acquisition and storage of excess tumor tissue to be used in the patient-direct xenograft program. The protocol was approved by The University of Texas MD Anderson Cancer Center Institutional Review Board under #LAB07-0854. Excess patient tumor was collected only after the planned surgical resection and pathologic examination were complete. Three patient specimens from pancreatectomies performed between 2009 and 2011 at The University of Texas MD Anderson Cancer Center were selected after reviewing medical records and tissue specimens.

**Cell lines and reagents**

Human pancreatic nestin-expressing (HPNE) cells, an hTERT-immortalized normal human ductal progenitor cell line [22], and 293T cells were obtained from Dr. Paul J. Chiao at The University of Texas MD Anderson Cancer Center at Houston, Texas. 293T and PANC-1 cell lines were obtained from ATCC (Manassas, VA). The established HPNE, PANC-1, and 293T cells were verified by DNA fingerprinting at the Characterized Cell Ductal Line Core Facility of the MD Anderson Cancer Center.

HPNE, PANC-1, and 293T cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies) at 37°C in a 5% CO₂ environment. Human recombinant TGF-β was purchased from R&D Systems (Minneapolis, MN).

**Plasmid transfection SMAD4**

Human retroviral short hairpin RNAi against human SMAD4 sequence 5’-GGTTGTCGATTGGAATGTA-3’ (shSMAD4) was identified from the He et al article [23], and the first 4 base pairs were replaced by AAAAA and used as a scrambled control SMAD4 short hairpin RNA (shScr). Its sequence was 5’-AAAATGCGATTGGAATGTA-3’. The pRetroSuper-GFP-shSMAD4 plasmid was purchased from Addgene (plasmid 15724; Cambridge, MA). shSMAD4 and shScr recombinant viruses were generated by transient transfection of the packaging plasmids pMLg/pRRE, pRSV.rev, and pHCMV-G into 293T cells. Virus-containing supernatant was collected after 72 hours to infect HPNE cells. The infected HPNE cells were purified by GFP fluorescence-activated cell sorting at the Flow Cytometry and Cellular Imaging Facility of MD Anderson. The effectiveness of SMAD4 knockdown was confirmed by western blotting and reverse-transcriptase (RT) polymerase chain reaction (PCR) analysis.

**Western blotting and immunofluorescence staining**

HPNE cells were harvested and solubilized in radioimmuno-precipitation assay protein lysis buffer (50 mM Tris HCL at pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mM EDTA, 1 mM sodium orthovanadate, 1 mM NaF, and 1x protease inhibitor cocktail) (Roche, Indianapolis, IN). Cell lysates (20 μg) were separated by electrophoresis on 8–10% SDS polyacrylamide gels, transferred to PVDF membranes (Millipore, Billerica, MA), and probed with different dilutions of antibodies of interest. The antibodies used in this study were against phospho-SMAD2 (Ser465/467), phospho-SMAD3 (Ser423/425), phospho-Akt (Ser473), phospho-MEK1/2 (Ser217/221), SMAD4, Taki1, SMAD2/3, MEK1/2, p21 Waf1/ Cip1 (all from Cell Signaling Technology, Danvers, MA), N-cadherin, vimentin, and cytokeratin 19 (CK19) (from Abcam, Cambridge, MA). Actin for protein loading control was purchased from Sigma-Aldrich (St. Louis, MO). Reactive bands were visualized with enhanced chemiluminescent reagents (GE Healthcare, Piscataway, NJ).

For TGF-β stimulation and phosphorylation studies, 90% confluent HPNE cells were washed twice with PBS and kept in serum-free medium overnight. Fresh 5% FBS medium was then added and the cells were incubated with recombinant TGF-β (5 ng/ml) for 2, 8, or 24 hours. Cell lysates were collected and immunoblotting with phospho-specific antibodies was used to analyze the various signaling pathways.

For immunofluorescence staining, HPNE cells were seeded into 8-well Lab-Tek II chamber slides (Thermo Fisher Scientific, Rochester, NY), fixed with 1% formalin the next day for 10 minutes at room temperature, and permeabilized with 1% Triton X-100 for 10 minutes. The chamber slides were then blocked with 1% BSA, and anti-N-cadherin and anti-Ck19 antibodies (5 ng/μl) were added and incubated overnight at 4°C. Secondary antibodies conjugated with Alexa Fluor 488 or Rhodamine Red-X (2 ng/μl) (Invitrogen Life Technologies, Grand Island, NY) were incubated with the slides for 30 minutes at room temperature, and the slides were counterstained with 4,6-diamidino-2-phenyindole.
Table 1. Primers used in this study for RT-PCR or real time PCR.

| Gene   | Forward (5'-3')       | Reverse (5'-3')       | Amplicon (bp) |
|--------|-----------------------|-----------------------|---------------|
| CDH1   | tgccgggaataaatgagaagg| gtgatgtggccagatggccct| 200           |
| CDH2   | ggcagctctccagagcga   | ggatgcctctcagatgctg  | 253           |
| VIM    | ggctcaggttgccaggcag  | gcttcaagcgcaagttctg  | 327           |
| BCA7   | atgctggtagcctctgacg | caagctctgctgcaacaa   | 220           |
| FN1    | caggtctttccacagtttaa | aagtctttgcctattgctggc | 277           |
| TWIST1 | acagctggtgctgctaag   | caagccctgtcttggtaaat | 291           |
| TWIST2 | agaagcagacatgtagcaca | gccatctctggggtgttg   | 327           |
| ZEB2   | gcctctggtgctgctaaagg| ttcctgggcagggacactac | 392           |
| GAPDH  | acggatctgttcttgtaag  | tgattttgaggggtctgc   | 200           |

RT-PCR and real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies) based on standard procedures. Complementary DNA was prepared with the iScript reverse transcription supermix kit (Bio-Rad Life Science, Hercules, CA) according to the manufacturer’s instructions. The primers were used in this study are listed in Table 1. The expression level of human CDH2 mRNA was quantified using iQ SYBR Green Supermix (Bio-Rad Life Science). Relative expression level was determined by normalizing the expression level of each target to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and relative mRNA fold change was determined using the 2(−ΔΔCt) method. Amplicons were run on a 2% agarose gel stained with ethidium bromide. The migration rate was determined as the ratio of the distance of the wound’s gap at 20 hours versus 0 hours using Adobe Photoshop software (San Jose, CA).

Modified Boyden chamber invasion and migration assays

A modified Boyden invasion chamber assay was used as previously described [24,25]. HPNE cells transfected with shSMAD4 (HPNE/shSMAD4 cells) and HPNE cells transfected with shScr (HPNE/shScr cells) were seeded onto serum-free medium in the top compartment of Matrigel-coated chambers (8.0-μm pores, BD Biosciences, Bedford, MA), where these cells were incubated with or without human TGF-β (10 ng/ml), and 20% FBS medium was added to the bottom compartment as a chemoattractant. Cells were allowed to invade across coated inserts for 20 hours. The cells on the apical surface of the insert were scraped off, and membranes with invaded cells were fixed in 100% methanol, stained with 1% crystal violet (Sigma-Aldrich), and mounted on slides. Experiments were performed for each condition and repeated three times.

Migration was demonstrated using 100-mm non-pyrogenic dishes [26]. Briefly, when HPNE/shScr and HPNE/shSMAD4 cells approached 100% confluence, the cell monolayer was scratched using a P200 micropipette tip. Cells were washed in PBS twice and incubated in 5% FBS medium for 20 hours with or without TGF-β (10 ng/ml) to allow them to migrate and close the wound. Three random images were taken at the time of the scratch and at 20 hours (Olympus IX71 inverted microscope, 4× magnification). The migration rate was determined as the ratio of distance of the wound’s gap at 20 hours versus 0 hours using Adobe Photoshop software (San Jose, CA).

Electrophoretic mobility shift assay

The CDH2 promoter fragment was examined for recognition by nuclear proteins by electrophoretic mobility shift assay. Nuclear protein was extracted from cells according to a protocol previously described [27]. Four oligo probes containing an SBE consensus binding sites were used (Table 2). The competitive binding experiment was performed with a 25-fold amount of oligonucleotides with wild-type and mutant SBE consensus binding sites. OCT-1 probe (5'-TGTGAATTGACAACTCAGTACCAAGG-3') was used as a loading control. The super-shift experiments were performed with anti-SMAD4 antibody (#SC-7966, Santa Cruz Biotechnology, Santa Cruz, CA). The four potential SBE sequences of the promoter and mutant oligos were purchased from Sigma (Sigma-Aldrich, Woodlands, TX).

Table 2. Primers used for CDH2 ChIP assay.

| Primers | Forward (5'-3')       | Reverse (5'-3')       | Tm (°C)  | Amplicon (bp) |
|---------|-----------------------|-----------------------|----------|---------------|
| A       | aatgctttcaacccctgga   | ttgttgttgagagatgtgc  | 59.99/60.04 | 238           |
| B       | tgcactttcaacccctgga   | ttcctggggagcctagta   | 59.84/59.84 | 290           |
| C       | cacggagaaacagttcaga   | acccttctacctcctccag  | 59.99/59.97 | 371           |

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Figure 1. Knocked down SMAD4 reduces N-cadherin protein level and inhibits invasion and migration in HPNE cells. (a) Western blot analysis of cell expression of SMAD4 and N-cadherin and CK19. Actin was used as the loading control. (b) RT-PCR results showing cell mRNA levels of CDH1, CDH2, CK19, VIM, BCAT, and FN1, and (c) TWIST1, TWIST2, SNAIL2, and ZEB1. GAPDH was used as the housekeeping control. (d) Immunofluorescence staining of CK19 and N-cadherin in HPNE/shScr or HPNE/shSMAD4 cells. CK19 and N-cadherin were labeled with red fluorescent Alexa Fluor 594 goat anti-rabbit IgG (A11012, Invitrogen). GFP-positive cells represent cells transfected with shScr or shSMAD4. Nuclei were counterstained with blue fluorescent 4,6-diamidino-2-phenylindole. Images were merged using Olympus CellSens software. (e) Modified Boyden chamber assay. HPNE/shScr and HPNE/shSMAD4 Cells were added with or without 10 ng/ml TGF-β to serum-free media inserts in the top chamber, and 20% FBS was placed in the bottom chamber as a chemoattractant. Invasive cells were counted in 3 fields at 10× magnification in duplicated inserts. (f) Wound-healing assay. HPNE/shScr and HPNE/shSMAD4 cells were treated with or without 10 ng/ml TGF-β. The y-axis represents cell...
Chromatin immunoprecipitation (ChIP)

ChIP assay was performed with a kit (EMD Millipore, Billerica, MA) as previously described [28]. Briefly, cells were cross-linked with 1% PFA, lysed, and sonicated, and the cell lysates were incubated with Protein A/G beads (Santa Cruz Biotechnology) with an anti-SMAD4 antibody (Cat #sc-7966X, Santa Cruz Biotechnology) overnight. DNA was reverse cross-linked and purified. Three real-time PCR primers (for CDH2 promoter sites A, B, and C, respectively) were used to amplify the corresponding region in the CDH2 promoter containing the three SBE binding sites. Primers used for real-time PCR are listed in Table 2.

CDH2 promoter luciferase reporter assay

The CDH2 promoter Firefly luciferase reporter gene was cotransfected into HPNE and 293T cells with an internal control, TK-Renilla luciferase, by using Lipofectamin2000 (#11668, Invitrogen). These cells were treated with TGF-β at different time points after 48 hours of transfection, and the activities of Firefly and Renilla luciferase were determined using a dual luciferase reporter assay system (#E4550, Promega, Madison, WI). Firefly luciferase activity was normalized to the Renilla luciferase activity of the internal control.

For site-directed mutagenesis assays, one base in the second SBE sequence in the CDH2 promoter region from −3795 bp to −3790 bp was changed using a mutagenesis kit (# 200522, Agilent Technologies, Inc., Santa Clara, CA). This SBE sequence of CAGACA was mutated into CACACA. The experiments were performed in triplicate.

Immunohistochemical staining

Tissues for this study were obtained from patients with primary pancreatic tumors and incorporated into our patient-direct xenograft program [29]. The tissues were embedded and cut into sections 4 µm thick for immunohistochemical study at the Clinical Core Laboratory of MD Anderson. Immunohistochemical staining for SMAD4 and N-cadherin was done using a Lab-Vision 480-2D immunostainer (ThermoFisher, Fremont, CA). All reactions were visualized with diaminobenzidine as a chromogen. Positive and negative controls were included in each run for each antibody used. Isotype controls for all antibodies were negative. Images were captured with an Olympus DP72 camera and CellSens software (Center Valley, PA) on an Olympus BX51 microscope at 10X magnification.

Statistical analysis

All quantified data were plotted and analyzed using GraphPad Prism 5.0 software (La Jolla, CA) with Student’s unpaired t-test or two-way ANOVA. Data are representative of at least 3 independent experiments and are reported as the mean ± s.d. of 3 replicates or triplicates unless otherwise indicated. A P value of <0.05 was considered statistically significant.

Results

Knocked down SMAD4 reduces N-cadherin protein level and inhibits invasion and migration in HPNE cells

To assess the role of SMAD4 in TGF-β-mediated EMT in pancreatic ductal cells, we used shRNA to silence SMAD4 expression [30] in HPNE cells. RT-PCR, immunoblotting, and immunofluorescence analyses demonstrated a decrease in the level of the mesenchymal protein N-cadherin and an increase in the epithelial marker cytokeratin 19 (CK19) (Figure 1a, 1b, and 1d). These results indicated that knock down of SMAD4 in HPNE cells not only down-regulated TGF-β-driven EMT, it also suggested a reversal of this process, known as mesenchymal-epithelial transition (MET) [14,31]. The EMT-upregulating transcription factors TWIST1 and ZEB1 were also partially suppressed after SMAD4 knockdown (Figure 1c). In contrast, knock down of SMAD4 in HPNE cells had no notable effect on the protein level of the mesenchymal protein vimentin or the epithelial protein β-catenin (data not shown). It also had no effect on the mRNA levels of CDH1, VIM, BCAT, and FNI. Western blot results demonstrated that proteins from the canonical TGF-β pathway, phospho-SMAD2 and phospho-SMAD3, and non-canonical pathways, such as phospho-AKT, phospho-MEK 1/2, and TAK1 showed such similar expression patterns after TGF-β treatment in HPNE, HPNE/shScr, and HPNE/shSMAD4 cell lines, suggesting they were unaffected (Figure S1a and 1b). p21, another downstream protein in the TGF-β pathway, showed the same pattern of expression in...
all three cell lines (Figure S1c). Cell proliferation assays indicated that these two cell lines have no significant cell growth alterations after exposure to TGF-β, as determined by MTT and colony formation assays (Figure S2a and S2b). TGF-β-induced EMT is known to regulate the migration and invasion capacity of epithelial cells. Control HPNE/shScr cells were less invasive and less migratory after exposure to TGF-β (p<0.001). Both invasion and migration were greatly diminished after knockdown of SMAD4 in the HPNE/shSMAD4 cells (p<0.001) (Figure 1e and 1f). The effect of SMAD4 knockdown on TGF-β-induced N-cadherin expression was most evident after exposure of HPNE cells to TGF-β in culture. HPNE/shSMAD4 cells demonstrated lower protein levels of N-cadherin during short-term culture. However, SMAD4 and N-cadherin expression in Figure 3. Map of multiple SBEs in CDH2 promoter. (a) Three SBEs with a CAGACA sequence (blue circles 1, 2, and 3) and one SBE with a GTCTAGAC sequence (red circle 4). Sections A, B, and C represent 3 primers and an amplifying region for ChIP assay in the promoter. (b) Electrophoretic mobility shift assay results showed that 4 SBE oligos had strong DNA and nuclear protein interaction bands (lane 1), binding was quenched by wild-type (WT) oligos (lane 2) and not by mutant (M) oligos (lane 3), and anti-SMAD4 antibody (Ab) inhibited binding activity (lane 4). (c) SBE binding activity was regulated by TGF-β treatment at 0, 2, 8, and 24 hours. Oct-1 DNA binding activities were determined as loading controls in (b) and (c). (d-f) ChIP assays and real-time PCR of primers A, B, and C comparing the ratio of IgG to anti-SMAD4 antibody with or without 5 ng of TGF-β at 0, 2, 8, and 24 hours, *p<0.05, **p<0.01, ***p<0.001.

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Table 3. Oligos from CDH2 promoter for EMSA.

| Oligos | Wildtype sequence (5'-3') | Mutant sequence (5'-3') | bp |
|--------|--------------------------|------------------------|----|
| 1      | cgagcgcctcagacaacaatagctae | cgagcgcctaaaacaacaatagctae | 26 |
| 2      | cgagcgcctcagacaacaatagctag | tcttaaaacaagtaatgctttgagca | 26 |
| 3      | aaagcactttttttaattgcagacae | aaagcactttttttaattgcaaaaae | 26 |
| 4      | ttctgtgtaaatttctctatttagctactt | ttctgtgtaaatttctctttaaagactt | 26 |

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HPNE/shSMAD4 cells was regained after continuous exposure to TGF-β over 5 days (Figure 2a). RT-PCR showed that CDH2 in HPNE/shSMAD4 cells was regained quickly, in 2 hours after TGF-β treatment (Figure 2b). Taken together, these data suggested that SMAD4 is a necessary component of the TGF-β-driven expression of N-cadherin in HPNE cells and that N-cadherin expression contributes to the EMT phenotype.

SMAD4 binds to SBEs located within the promoter region of CDH2

On the basis of our preliminary observations (Figures 1 and 2), we therefore searched the region of the CDH2 gene and identified four candidate SBE sequences located at three sites within the promoter (−1980, −3795, and −5620 bp). These sites may cooperate to regulate CDH2 transcription (Figure 3a). Physical binding of SMAD4 to the sites was demonstrable by EMSA and

**Figure 4. TGF-β Stimulated CDH2 promoter Dual-luciferase Activity.** (a) Schematic of five pGL2-CDH2 promoter constructs, including part of the CDH first exon (white rectangles), and potential SBE sites (downward arrows). (b,c) Luciferase activity ratios of CDH2 clone #5 and F1/R1 construct treated with or without 5 ng of TGF-β at 0, 2, 8, and 24 hours in HPNE and 293T cells. **P < 0.01, ***P < 0.001. (d) The second SBE sequence in the CDH2 promoter and the Mut2 mutant sequence (G→C). (e) Luciferase activity ratio of Mut2 with or without 5 ng TGF-β at 0, 2, 8, and 24 hours in HPNE and 293T cells.

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Scheme of pGL2-CDH2 promoter constructs. Clone #5 and F1/R1 have been tested with luciferase assay. ↓ part of CDH2 first exon, ▽: potential Smad binding sites.
ChIP assays. All four oligos have specific physical binding to HPNE nuclear protein. This binding was only blocked by wild type oligos but not by mutant oligos, and this binding was inhibited by a SMAD4 antibody in vitro (Figure 3a). The sequences of these wildtype and mutant oligos are listed in Table 3. When these cells were exposed to TGF-β, this binding was generally increased at 2 and 8 hours. These assays confirmed that exposure to TGF-β enhanced binding of the oligos to SBEs in the CDH2 promoter (Figure 3c). ChIP assay results demonstrated that replicons from primers A, B, and C were increased at 2 hours (p < 0.05) and 8 hours (p < 0.01 and 0.001) after TGF-β treatment as determined by comparing signal from a SMAD4 antibody and an IgG control (Figure 3d, 3e, and 3f). Therefore, this ChIP assay corroborated the specific binding on the CDH2 promoter as seen by EMSA. To check whether this binding can be generalized to pancreatic cancer cells, we used oligo 2 to perform EMSA in PANC-1 cells after TGF-β exposure. Results showed specific binding of oligo 2 to PANC-1 nuclear protein in a similar pattern as HPNE cells. This binding was decreased in 2 hours after TGF-β treatment. The binding was competed and inhibited by wildtype oligo 2 and SMAD4 antibody respectively, and not by mutant oligo 2 (Figure S3).

TGF-β stimulated CDH2 promoter dual-luciferase activity

Our results are the first to demonstrate that the presence of these SBEs is necessary for TGF-β-induced transcription, we were utilized luciferase reporter constructs within HPNE and 293T cells. Five different constructs were cloned into pGL2-Basic vectors (Promega) (Figure 4a). Within HPNE cells, the highest levels of luciferase expression occurred when all four SBEs were intact (construct #5) suggesting that they all cooperate to enhance transcription of CDH2. Two SBEs (CAGACA) nearest to the CDH2 coding region (construct F1/R1) were necessary for any expression to occur at baseline or after TGF-β stimulation in both HPNE and 293T cells. Luciferase activity was increased significantly at 2 hours with and without TGF-β treatment (construct #5, p < 0.05) (Figure 4b), and again on construct F1/R1 at 2 hours and 8 hours (p < 0.01 and 0.001; Figure 4c). The results suggested that the second SBE on construct F1/R1 was the most important for TGF-β stimulation of luciferase activity. The other three constructs (4a, F2/R3, F2/R2) produced no luciferase activity. To verify the importance of the second SBE on construct F1/R2, we specifically mutated the construct at 23780 from CAGACA to CACACA This caused luciferase activity to be significantly reduced with and without TGF-β treatment, thereby demonstrating that binding at this site is a vital step for CDH2 transcription (Figure 4d and 4e).

Loss of CDH2 transcription and N-cadherin expression recapitulates the HPNE/shSMAD4 cell phenotype

We next sought to confirm whether loss of expression of the SMAD4-controlled target gene, CDH2, could produce the same...
cellular phenotype observed in SMAD4-knockdown HPNE cells. When shCDH2 was introduced to cells, production of N-cadherin and transcription of CDH2 were inhibited as demonstrated by western blot (Figure 5a), and both RT-PCR (left) and real time PCR (right) (Figure 5b) in a pattern very similar to that observed in our preceding experiment. HPNE/shCDH2 cells demonstrated decreased invasive capacity and migration when compared to HPNE/shScr control cells (p<0.001) (Figure 5c and 5d).

SMAD4 Regulates N-Cadherin in Human Pancreatic Ductal Epithelium

Figure 6. SMAD4 and N-cadherin expression levels in patient tumors and matched xenograft samples. (a) Representative immunostained images of patients’ primary tumors and (b) and matched patient-direct xenograft tumors (PATX). Images were captured with an Olympus DP72 camera (10 × magnification). (c) Western blotting results for corresponding patient-direct xenograft tumor lysates. Actin was used as the loading control.

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SMAD4 and N-cadherin immunohistochemistry staining on patient specimens

We immunohistochemically stained specimens from 3 patients and matched xenograft samples and observed the correlation of SMAD4 and N-cadherin staining. The high expression of SMAD4 in tissue from patient 70 and in its derived xenograft PATX70 correlated with high N-cadherin expression, whereas the low expression of SMAD4 in tissues from patients 39 and 52 and in
SMAD4 can change a patient’s local tumor environment through maintaining N-cadherin expression, and that loss or mutation of SMAD4 can change a patient’s local tumor environment through altering the N-cadherin level.

Discussion

It is well known that EMT has a tremendous role in tumor invasion, metastasis, generating cancer stem cells, and drug resistance from in vitro and in vivo pancreatic cancer studies [32]. This phenotype is characterized by increased N-cadherin expression, whose functions have been extensively studied in cervical cancers [18–21].

In conclusion, these findings describe a TGF-beta signaling pathway that is necessary for functional characterization of a Smad binding element (SBE) in the JunB promoter, through which SMAD4 regulates N-cadherin transcription in normal pancreatic ductal epithelial cells.

Supporting Information

Figure S1 Changes in TGF-β canonical and non-canonical pathways in HPNE, HPNE/shScr, and HPNE/shSMAD4 cells. Western blots of cells 2, 4, 8, and 24 hours after treatment with 5 ng/ml TGF-β. (a) Phospho-SMAD2 and phospho-SMAD3 protein expression levels. Total SMAD2/3 was used as the loading control, (b) Phospho-Akt, phospho-1/2Mek, and TAK1 protein expression levels. Respective loading controls were total Akt, 1/2 Mek, and actin. (c) p21 Protein expression levels. Actin was used as the loading control.

(TIF)

Figure S2 Cell proliferation and colony formation assays of HPNE, HPNE/shScr, and HPNE/shSMAD4 cells. (a) Cells were seeded at 1000 cells per well in 96 wells in triplicate and treated with fresh TGF-β (5 ng/ml) every other day, MTT assay was performed according to the manufacturer’s recommendation. Absorbance was determined at 570 nm at days 1, 3, 5, and 7 using a microplate reader (FLUOstar Omega, BMG Labtech, Chicago, IL). (b) Cells were plated at 500 cells per 60-mm dish in triplicate and treated with fresh TGF-β (5 ng/ml) every other day and incubated for 14 days. The cells were then fixed and stained with 0.5% crystal violet in methanol. The number of colonies was counted manually.

(TIF)

Figure S3 Electrophoretic mobility shift assay in PANC-1 cells. (a) SBE oligo 2 had strong DNA and nuclear protein interaction bands (lane 1). Binding was quenched by wild-type (WT) oligo 2 (lane 2) and not by mutant (M) oligo 2 (lane 3). Anti-SMAD4 antibody (Ab) inhibited binding activity (lane 4). (b) SBE binding activity was regulated by TGF-β treatment at 0, 2, 8, and 24 hours. Free probe activities were determined as loading controls in (a) and (b).

(TIF)

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Author Contributions

Conceived and designed the experiments: YK PJC JBF. Performed the experiments: YK JL RS XCL. Analyzed the data: MJT RMT MHK. Contributed reagents/materials/analysis tools: DC HW. Wrote the paper: YK DR JBF.

References

1. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144: 646-674.
2. Riim AD, Mirek ET, Aiello NM, Maitra A, Bailey JM, et al. (2012) EMT and dissemination precede pancreatic tumor formation. Cell 148: 349-361.
3. Katz MH, Wang H, Fleming JB, Sun CC, Hwang RF, et al. (2009) Long-term survival after multidisciplinary management of resected pancreatic adenocarcinoma. Ann Surg Oncol 16: 836-847.
4. Jonk LJ, Itoh S, Heldin CH, ten Dijke P, Kruijer W (1998) Identification and characterization of a membrane protein-inducible enhancer. J Biol Chem 273: 8361-847.
5. Shi Y, Wang YF, Jayaraman L, Yang H, Massague J, et al. (1998) Crystal structure of a Smad binding element domain bound to DNA: insights on DNA binding in TGF-beta signaling. Cell 94: 505-514.
6. Zawel L, Dai JL, Buckhaults P, Zhou S, Kinzler KW, et al. (1998) Human Smad3 and Smad4 are sequence-specific transcription activators. Mol Cell 1: 611-617.
7. Massague J, Wotton D (2000) Transcriptional control by the TGF-beta/Smad signaling system. EMBO J 19: 1745-1754.
8. Massague J (2008) TGFbeta in Cancer. Cell 134: 215-230.
9. Pickup M, Novitskiy S, Moses HL (2013) The roles of TGFbeta in the tumour microenvironment. Nat Rev Cancer 13: 78-99.
10. Hall SA, Schutte M, Hoque AT, Moskaluk CA, da Costa LT, et al. (1996) DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science 271: 350-353.
11. Schutte M, Hruban RH, Hedrick L, Cho KR, Nadasdy GM, et al. (1996) DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science 271: 350-353.
12. Hay ED (1995) An overview of epithelial-mesenchymal transformation. Acta Anat (Basel) 154: 8-20.
13. Perez-Pomares JM, Munez-Chapado R (2002) Epithelial-mesenchymal transition: a mesodermal cell strategy for adaptive evolution in Metasanz. Anat Rec 268: 343-351.
14. Thiery JP, Sleeman JP (2006) Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol 7: 131-142.
15. Bardeesy N, Cheng KH, Berger JH, Chu GC, Palher J, et al. (2006) Smad4 is dispensable for normal pancreas development yet critical in progression and tumor biology of pancreas cancer. Genes Dev 20: 3130–3146.

16. Takeichi KHaM (1986) Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development. Nature 320: pp. 447–449.

17. Bidhy JL, Zhang R (1990) Purified N-cadherin is a potent substrate for the rapid induction of neurite outgrowth. J Cell Biol 110: 1253–1260.

18. Giorgi LA, Bringuier PF, Shimazui T, Jansen K, Schalken JA (1999) Changes in cadherin-catenin complexes in the progression of human bladder carcinoma. Int J Cancer 82: 70–76.

19. Rieger-Christ KM, Pezza JA, Daugan JM, Braasch JW, Hughes KS, et al. (2001) Disparate E-cadherin mutations in LCIS and associated invasive breast carcinomas. Mol Pathol 54: 91–97.

20. Hazan RB, Phillips GR, Qiao RF, Norton L, Aaronson SA (2000) Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. J Cell Biol 148: 779–790.

21. Shintani Y, Hollingsworth MA, Wheelock MJ, Johnson KR (2006) Collagen I promotes metastasis in pancreatic cancer by activating c-Jun NHE2-terminal kinase 1 and up-regulating N-cadherin expression. Cancer Res 66: 11754–11758.

22. Lee KM, Nguyen C, Ulrich AB, Pour PM, Ouellette MM (2005) Immortalization with telomerase of the Nestin-positive cells of the human pancreas. Biochem Biophys Res Commun 303: 1038–1044.

23. He W, Dorn DC, Erdjument-Bromage H, Tempst P, Moore MA, et al. (2006) Hematopoiesis controlled by distinct TIF1gamma and Smad4 branches of the TGFbeta pathway. Cell 125: 929–941.

24. Li Z, Chang Z, Chiao LJ, Kang Y, Xia Q, et al. (2009) TrkBT1 induces liver metastasis of pancreatic cancer cells by sequestering Rho GDP dissociation inhibitor and promoting RhoA activation. Cancer Res 69: 7851–7859.

25. Thomas RP, Kocher M (2007) Percutaneous treatment of benign biliary strictures and biliary manometric perfusion test. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 151: 85–90.

26. Chun-Chi Liang AYPJ-LG (2007) In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nature Protocols 2: 329–333.

27. Leng J, Kang Y, Zhao R, Xia Q, Lee DF, et al. (2012) KraG12D-induced IKK2/beta/NF-kappaB activation by IL-1alpha and p62 feedforward loops is required for development of pancreatic ductal adenocarcinoma. Cancer Cell 21: 105–120.

28. Hirschtritt ME, Pagano ME, Christian KM, McNamara NK, Stanaberry RJ, et al. (2012) Modulators of flutaxine treatment response for children and adolescents with comorbid depression and substance use disorders. J Subst Abuse Treat 42: 366–372.

29. Kim MP, Evans DB, Wang H, Abbruzzese JL, Fleming JB, et al. (2009) Generation of orthotopic and heterotopic human pancreatic cancer xenografts in immuno-deficient mice. Nat Protoc 4: 1670–1680.

30. Padillo R, Caudy AA, Bernstein E, Hannon GJ, Coulkin DS (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev 16: 948–958.

31. Thiery JP, Acoque H, Huang YR, Nieto MA (2009) Epithelial-mesenchymal transitions in development and disease. Cell 139: 871–890.

32. Cano CE, Motoo Y, Iovanna JL (2010) Epithelial-to-mesenchymal transition in pancreatic adenocarcinoma. ScientificWorldJournal 10: 1947–1957.

33. LeFort CT, Wegrzenczuk K, Hocking DC (2011) N-cadherin cell-cell adhesion complexes are regulated by fibronectin matrix assembly. J Biol Chem 286: 3149–3160.

34. Sun Z, Patrich AR, Hill MA, Meininger GA (2014) N-cadherin, a vascular smooth muscle cell-cell adhesion molecule: Function and signaling for vasomotor control. Microcirculation.

35. Shih W, Yamada S (2012) N-cadherin-mediated cell-cell adhesion promotes cell migration in a three-dimensional matrix. J Cell Sci 125: 3661–3670.