

Loss of FOXA1/2 Is Essential for the Epithelial-to-Mesenchymal Transition in Pancreatic Cancer

Yan Song¹, M. Kay Washington², and Howard C. Crawford¹

Abstract

FOXA1 and FOXA2, members of the forkhead transcription factor family, are critical for epithelial differentiation in many endoderm-derived organs, including the pancreas. However, their role in tumor progression is largely unknown. Here, we identified FOXA1 and FOXA2 as important antagonists of the epithelial-to-mesenchymal transition (EMT) in pancreatic ductal adenocarcinoma (PDA) through their positive regulation of E-cadherin and maintenance of the epithelial phenotype. In human PDA samples, FOXA1/2 are expressed in all epithelia from normal to well-differentiated cancer cells, but are lost in undifferentiated cancer cells. In PDA cell lines, FOXA1/2 expression is consistently suppressed in experimental EMT models and RNAi silencing of FOXA1/2 alone is sufficient to induce EMT. Conversely, ectopic FOXA1/2 expression can potentially neutralize several EMT-related E-cadherin repressive mechanisms. Finally, ectopic FOXA2 expression could reactivate E-cadherin expression in a PDA cell line with extensive promoter hypermethylation. In fact, demethylation-mediated reactivation of E-cadherin expression in these cells required concurrent reactivation of endogenous FOXA2 expression. We conclude that suppression of FOXA1/2 expression is both necessary and sufficient for EMT during PDA malignant progression. *Cancer Res*; 70(5); 2115–25. ©2010 AACR.

Introduction

The epithelial-to-mesenchymal transition (EMT) is a physiologic process, originally described in embryonic development, in which cells lose epithelial characteristics and gain mesenchymal properties. EMT is accompanied by loss of cell-to-cell contact and increased cell motility. It is also implicated in late-stage tumor progression as a prelude to cancer metastasis (1, 2). As a gatekeeper of the epithelial phenotype (2), E-cadherin regulation has been extensively studied during EMT, with most research focusing on its transcriptional repression by E12/47, TWIST (3), and members of the Snail (4, 5) and ZEB (6) protein families. In contrast, little attention has been paid to the involvement of E-cadherin transcriptional activators in EMT because repressive factors are thought to be dominant.

FOXA proteins belong to subclass A of the forkhead box containing transcription factor family (Fox proteins). FOXA1 and FOXA2 share 92% homology within their forkhead DNA-binding domains (7). Genetic ablation of *Foxa1* or *Foxa2* in

mice causes postnatal or embryonic lethality, respectively (8–11). Individual or compound deletions of *Foxa1* and *Foxa2* show that both *Foxa1* and *Foxa2* are essential for terminal differentiation and maturation of many endoderm-derived cells, including α -cells in the endocrine pancreas (12) and liver (13), lung alveolar (14), and prostate luminal ductal epithelia (15). These findings suggest that *Foxa1/2* proteins are critical for both early embryonic development and late- or end-stage epithelial differentiation.

Little is known about the role of FOXA1/2 in cancer even though their expression is observed in many human cancers including prostate (16), breast (17, 18), lung, and esophagus (19). The involvement of FOXA proteins in pancreatic ductal adenocarcinoma (PDA) has yet to be described. *Foxa1* and *Foxa2* are important transcription factors for endocrine pancreas development and function (7) and are expressed in exocrine pancreas where their function is unclear (20, 21).

PDA has a 5-year survival rate of less than 4%, a statistic largely attributable to its aggressive invasive and metastatic behavior. We found that both FOXA1 and FOXA2 were expressed in most stages of PDA progression, but were lost in undifferentiated cancer cells. In PDA cell lines, FOXA1/2 expression was suppressed in experimental EMT models, and forced inhibition of FOXA1/2 factors was sufficient to induce EMT. As activators of E-cadherin transcription, FOXA1/2 overexpression could overcome several repressive signals of E-cadherin expression, including Snail1 overexpression, transforming growth factor- β (TGF β) treatment, and E-cadherin promoter hypermethylation. Interestingly, the *FOXA2* gene itself was suppressed by DNA methylation, and this repression was critical for methylation-mediated silencing of E-cadherin gene expression. Taken together,

Authors' Affiliations: ¹Department of Pharmacology, Stony Brook University, Stony Brook, New York and ²Department of Pathology, Vanderbilt University Medical Center, Nashville, Tennessee

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Corresponding Author: Howard C. Crawford, Department of Pharmacology, Stony Brook University, 1 Circle Road, BST 8-140, Stony Brook, NY 11794-8651. Phone: 631-444-3085; Fax: 631-444-9749; E-mail: crawford@pharm.stonybrook.edu.

doi: 10.1158/0008-5472.CAN-09-2979

©2010 American Association for Cancer Research.

our study shows that loss of FOXA1/2 is a critical event in EMT during pancreatic cancer progression.

Materials and Methods

Human tissue and immunohistochemistry. Sections of archival formalin-fixed, paraffin-embedded human PDA and pancreatitis samples were obtained from the Tissue Core, Vanderbilt University Medical Center. Primary antibodies used for immunohistochemical analysis were anti-FOXA1 (Santa Cruz Biotechnology, Inc.) and anti-FOXA2 (Seven Hills Bioreagents). Hematoxylin was used at 1:10 dilution as counterstain to avoid interference with nuclear FOXA1/2 staining. Images were captured on an Olympus U-DO3 microscope.

Cell culture and treatments. Human PDA cell lines were purchased from the American Type Culture Collection (ATCC) and maintained at 37°C in 5% CO₂ in ATCC recommended medium supplemented with 10% fetal bovine serum (FBS) and 0.5 µg/mL gentamicin. Phase-contrast images were captured on a Zeiss Axiovert 200M microscope. Immunofluorescence images were captured on a Zeiss LSM-510 Meta confocal microscope. In indicated experiments, cells were treated with 5 ng/mL TGFβ (R&D Systems), 20 ng/mL hepatocyte growth factor (HGF; R&D Systems), 5'-aza-deoxycytidine (5'-aza-dC; Sigma-Aldrich), or their corresponding vehicle controls. See Supplementary Data for antibodies, primers, and other methods.

Plasmids, siRNA, shRNA, and generation of stable cells. Expression vectors for mouse *Foxa1* and rat *Foxa2* were gifts of Dr. Robert Matusik (Vanderbilt University, Nashville, TN). Expression vector of *Snail1* (pCDNA3.1/GS-Snail1) was purchased from Invitrogen. FOXA1-specific or FOXA2-specific siRNA (ON-TARGETplus, Dharmacon; see Supplementary Table S2 for sequences) was transfected using TransIT-TKO (Mirus). A scrambled siRNA sequence (BLOCK-iT Alexa Fluor fluorescent, Invitrogen) was used as control siRNA and as an indicator of transfection efficiency. Lentiviral-based shRNA constructs (pLKO.1-puro) targeting either the FOXA1 or the FOXA2 gene were purchased from Sigma (see Supplementary Table S2 for sequences). Control pLKO.1 vector was a gift from Dr. Holly Colognato (Department of Pharmacology, Stony Brook University, Stony Brook, NY). Freshly prepared viruses were used to infect BxPC3 cells, which were then selected and maintained with 0.8 µg/mL puromycin. The coding sequence of rat *Foxa2* was subcloned into the pMIG retrovirus vector with an internal ribosome entry site (IRES)-green fluorescent protein (GFP). Viruses containing either *Foxa2*-IRES-GFP or control IRES-GFP were packaged in amphi-Phoenix cells, and GFP⁺ PANC-1 cells were enriched by fluorescence-activated cell sorting (FACS).

Promoter constructs and luciferase reporter assay. A 921-bp human *E-cadherin* promoter region was subcloned into pGL3 Basic vector (Promega). Three FOXA binding elements were mutated according to a previous report (22). Promoter activity was measured with the Dual Luciferase Kit (Promega). See Supplementary Data for details.

Migration assay. Cells were seeded onto Transwell membrane inserts (8 µm; Corning) in serum-free medium.

Medium containing 10% FBS was added to the lower chamber. After 6-h incubation at 37°C, cells that migrated to the lower surface of the membrane were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet. When siRNA-mediated FOXA1/2 silencing was used, cells were assessed 48 h after transfection. For each membrane, five random fields were counted at ×10 magnification. The mean was calculated and data were presented as mean ± SEM from three independent experiments done in triplicate.

Statistics. Data were presented as mean ± SEM. Student's *t* test was used in two-group comparisons. *P* < 0.05 was considered statistically significant. Expression of FOXA1/2 in PDA was analyzed by χ^2 test.

Results

Expression of FOXA1/2 is lost in undifferentiated PDA cells. The critical function of FOXA1/2 in pancreas development and in several types of cancer led us to examine their expression in human PDA using immunohistochemistry. Twenty-five human PDA and five chronic pancreatitis samples contained multiple epithelial pathologies, including metaplasia, pancreatic intraepithelial neoplasia (PanIN), differentiated invasive PDA, and undifferentiated invasive PDA. Results are summarized in Supplementary Table S1, divided by pathology, as found in each sample.

In nearby normal tissue, FOXA1 and FOXA2 were expressed in all islet cell types and at lower levels in acinar and ductal cells, primarily localized to the nucleus (Fig. 1A), a pattern consistent with that found in mouse pancreas (20, 21). Among the 25 PDA specimens, 24 contained invasive cancer and 12 contained various stages of PanIN. FOXA1 and FOXA2 localized primarily to the nucleus and were expressed in invasive adenocarcinoma cells in 79% (19 of 24) and 75% (18 of 24) of PDA samples (Fig. 1B), respectively, although strong cytoplasmic staining was observed occasionally for FOXA1/2 (3 of 24) in poorly differentiated cancers. Both FOXA1 and FOXA2 were detected in 83% (10 of 12) of PanINs at all stages (Fig. 1A). FOXA1 and FOXA2 were also expressed in all identifiable ductal metaplasia associated with PDA (25 of 25) and in 5 of 5 chronic pancreatitis cases examined (Fig. 1A).

Strikingly, very few poorly differentiated cancer cells maintained FOXA1 (26%, 5 of 19) or FOXA2 (11%, 2 of 18) expression, in contrast to well-differentiated cancer cells in the same sections (Fig. 1B; Supplementary Table S1). This was especially evident in isolated invasive anaplastic cancer cells, which commonly showed no expression of FOXA1 or FOXA2, in contrast to the nuclear expression of FOXA1 or FOXA2 in nearby moderately well-differentiated carcinomas (Fig. 1B). Thus, FOXA1/2 were expressed in normal pancreas epithelium and maintained in metaplastic epithelium, PanIN lesions, and well-differentiated PDA, but were lost in poorly differentiated cancer cells.

Expression of FOXA1/2 factors in PDA cell lines and experimental EMT. Loss of FOXA1/2 expression in poorly differentiated PDA provoked the question whether these factors play a role in EMT. To begin to address this question, a cohort of pancreatic cancer cell lines were examined for the

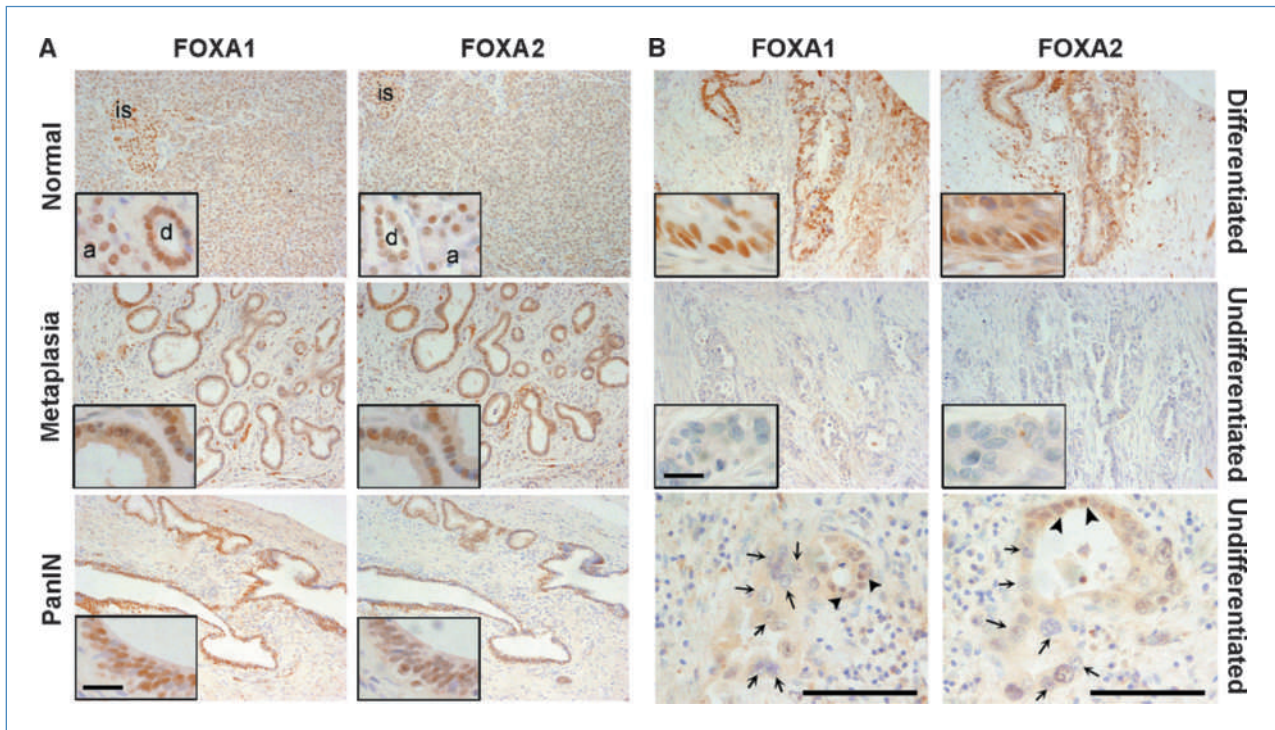


Figure 1. FOXA1 and FOXA2 expression in human PDA samples. Human PDA samples were assessed for FOXA1 and FOXA2 by immunohistochemistry of nearby normal epithelia, metaplasia, and PanIN lesions (A) and of well-differentiated or undifferentiated PDA (B). Note strong nuclear staining for FOXA1/2 in all the stages except in undifferentiated PDA. Undifferentiated cancer cells with large atypical nuclei (arrows) were consistently negative for both FOXA1 and FOXA2, in contrast to the positive staining of nearby moderately differentiated cancer cells (arrowheads). Bar, 100 μ m (main pictures) and 50 μ m (insets). Representative images are shown from 24 human PDA specimens. is, islet; a, acinar; d, duct.

expression of FOXA1/2 and EMT-related genes, including E-cadherin, vimentin, Snail1, and Slug, by quantitative reverse transcription-PCR (RT-PCR; Supplementary Fig. S1) and Western blot analysis (Fig. 2A). FOXA1 and FOXA2 expression was found in every cell line with the exception of MiaPaCa-2 cells, an E-cadherin-negative, vimentin-positive line (Fig. 2A). Even though E-cadherin transcriptional repressors, such as Snail1, have been characterized as dominant mediators of the undifferentiated status in a variety of cancer cell lines, including MiaPaCa-2 (4, 23), PDA lines characterized as moderately differentiated, CFPAC-1 and PANC-1 (24), expressed levels of Snail1 or Slug comparable to those in MiaPaCa-2 cells (Fig. 2A; Supplementary Fig. S1).

To test if FOXA1/2 factors were involved in EMT, we used two experimental models of EMT. PANC-1 and HPAFII are well-documented PDA cell lines that undergo EMT on TGF β (25, 26) or HGF stimulation (27), respectively. EMT was induced in both cell lines within 48 hours of the respective treatments, illustrated by loss of E-cadherin and gain of vimentin expression (Fig. 2B), although an increase of vimentin protein was undetectable in HPAFII cells. FOXA1 and FOXA2 mRNA and protein were consistently suppressed in both model systems (Fig. 2C and D), suggesting that downregulation of FOXA1/2 factors was part of the EMT program, in coordination with upregulation of E-cadherin transcriptional repressors such as Snail1 and Slug (Fig. 2C and D).

Loss of FOXA1/2 induces EMT. We next tested whether direct inhibition of FOXA1/2 alone could induce EMT. Two well-differentiated PDA cell lines, HPAFII (Fig. 3) and BxPC3 (Supplementary Fig. S2A and B), were transiently transfected with synthetic siRNAs against either FOXA1 or FOXA2. Whereas FOXA1/2 were inhibited to 50% to 60% of control levels, E-cadherin expression was diminished at both mRNA and protein levels (Fig. 3A and B). Knockdown of FOXA1 and FOXA2 together further reduced E-cadherin expression by 80%, indicating that the total quantity of FOXA proteins was critical for optimal E-cadherin expression. Consistent with a previous report indicating that FOXA2 is essential for FOXA1 expression (28), FOXA1 was consistently inhibited when FOXA2 was silenced, possibly explaining why FOXA2 silencing generally had a stronger effect than FOXA1 silencing (Fig. 3A and B). Localization of E-cadherin was unchanged, remaining at cell-cell junctions (Fig. 3C). Transcripts for ZO-1, another epithelial marker, were also inhibited as FOXA1/2 factors were suppressed (Fig. 3B), supporting a loss of epithelial character. However, neither the mesenchymal markers vimentin and fibronectin nor E-cadherin-repressing Snail transcription factors were significantly upregulated (Fig. 3B), indicating that transient FOXA1/2 inhibition could initiate the loss of epithelial traits but was not sufficient to induce the mesenchymal phenotype.

To study the long-term effects of FOXA1 or FOXA2 silencing, stable lines were established from BxPC3 cells that expressed shRNAs against either *FOXA1* or *FOXA2*. BxPC3 is a well-differentiated PDA cell line with abundant expression of E-cadherin and FOXA1/2 (Fig. 2A). In agreement with transient knockdown experiments, inhibition of either FOXA1 or FOXA2 alone impaired endogenous E-cadherin expression, with simultaneous knockdown of both FOXA1 and FOXA2 being more effective (Fig. 4A). In contrast to transient knockdowns, transcripts for vimentin, fibronectin, and Snail1

were significantly elevated (Fig. 4B). Morphologically, cells lacking FOXA1 or FOXA2 showed an increase in cell spreading, exhibiting a spindle shape and reduced cell-cell contact (Fig. 4C) compared with control cells. Functionally, cell migration was enhanced 4- to 6-fold when either FOXA1 or FOXA2 was silenced and by ~10-fold when both FOXA1/2 factors were silenced, as assessed by Transwell migration assay (Fig. 4D). Cell proliferation was also moderately inhibited in FOXA1- or FOXA2-deficient BxPC3 cells (Supplementary Fig. S2). Collectively, these findings indicate that FOXA1/2

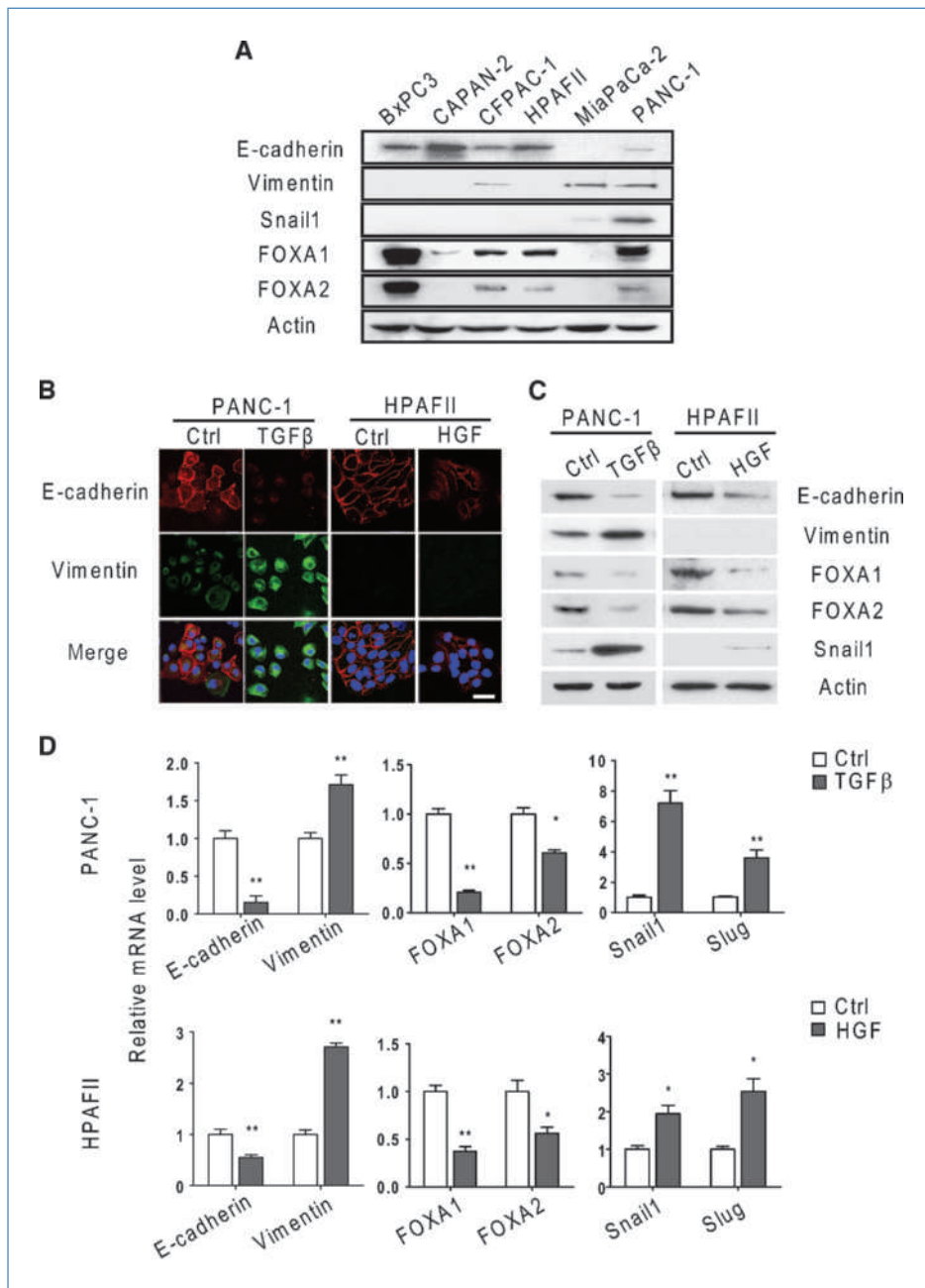
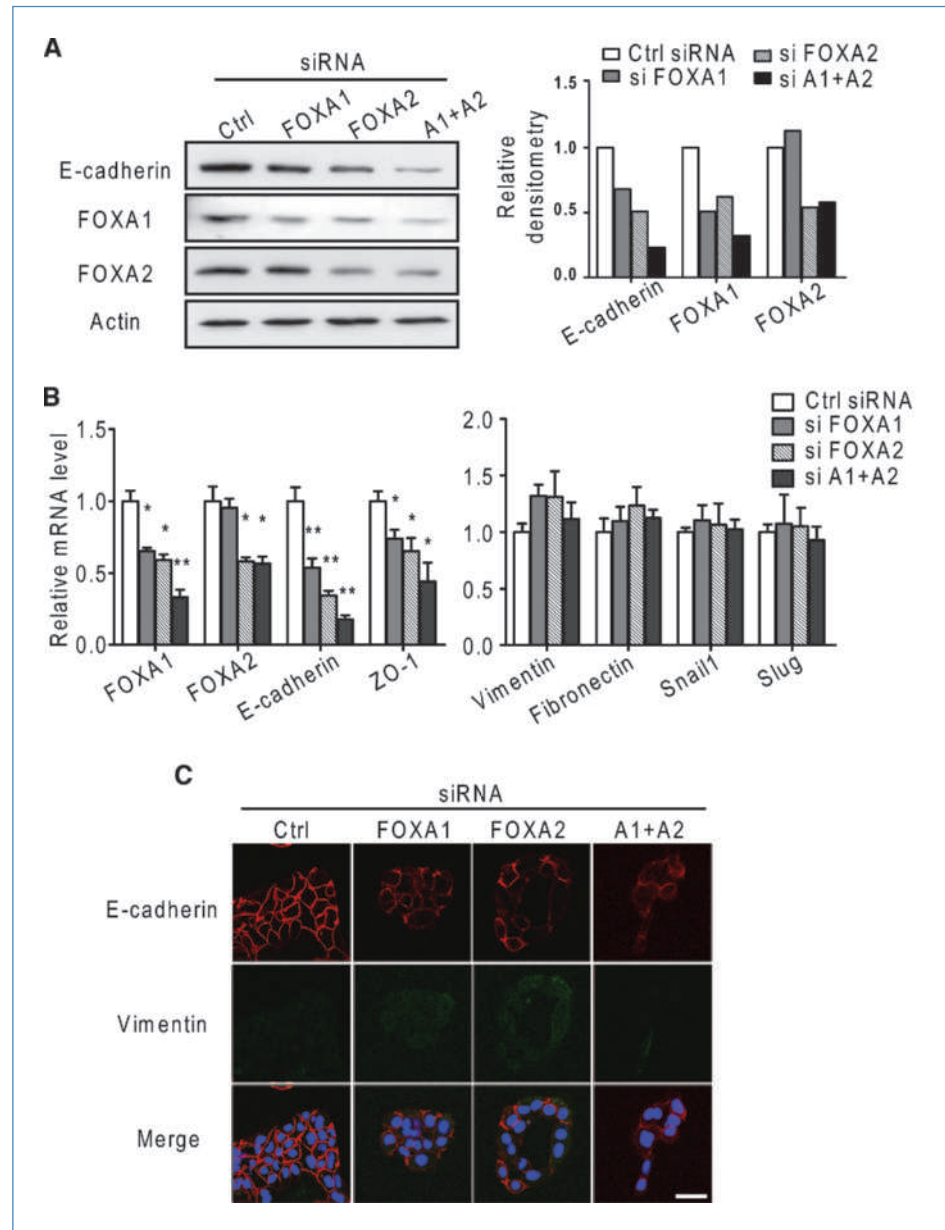


Figure 2. FOXA1 and FOXA2 are suppressed during EMT *in vitro*. A, expression of E-cadherin, vimentin, Snail1, FOXA1, and FOXA2 was analyzed by Western blot analysis in human PDA cell lines (A). B to D, PANC-1 or HPAFII cells were treated for 48 h with 5 ng/mL TGFβ or 20 ng/mL HGF, respectively, to induce EMT. B, immunofluorescence staining confirmed the repression of E-cadherin (red) and the activation of vimentin (green). 4',6-Diamidino-2-phenylindole staining is shown in blue. Bar, 100 nm. C and D, expression of EMT-related genes was determined by Western blot analysis (C) and quantitative RT-PCR (D). Actin was used as a loading control in the Western blots. A representative Western blot is shown from three independent experiments. Glyceraldehyde-3-phosphate dehydrogenase served as a normalization control for quantitative RT-PCR. Columns, mean from three independent experiments; bars, SEM. *, $P < 0.05$; **, $P < 0.01$.

Figure 3. Transient inhibition of FOXA1/2 leads to loss of epithelial markers. HPAFII cells were transiently transfected with siRNA against either FOXA1 or FOXA2, or a combination of both. A, expression of E-cadherin and FOXA1/2 was examined 48 h after siRNA transfection. Quantitation of the representative blot is shown in the bar graph (right). B, expression of FOXA1/2, E-cadherin, ZO-1, vimentin, fibronectin, Snail1, and Slug was examined by quantitative RT-PCR 48 h after siRNA transfection. C, representative immunofluorescence staining of E-cadherin 60 h after transfection. Bar, 100 nm. Representative data are shown in A and C from three independent experiments. Means \pm SEM are shown for B from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$.



factors are essential in maintaining the epithelial phenotype, with their long-term loss being sufficient to induce a secondary mesenchymal transition.

FOXA2 can overcome suppressive signals to activate the E-cadherin promoter. To further understand the relationship between FOXA1/2 factors and epithelial differentiation in PDA cells, we examined direct regulation of the E-cadherin promoter by these transcription factors. In both MiaPaCa-2 (data not shown) and PANC-1 cells (Fig. 5A), FOXA2 overexpression activated an E-cadherin promoter/luciferase reporter in a dose-dependent (Fig. 5A) and FOXA binding site-dependent (Supplementary Fig. S3A and B) manner. Even though FOXA1 seemed to contribute to endogenous E-cadherin expression (Figs. 3 and 4), its overexpression

did not activate the E-cadherin promoter/reporter in either cell line. Nevertheless, chromatin immunoprecipitation assays showed that endogenous FOXA1 and FOXA2 proteins interacted with DNA regions that included proximal consensus FOXA binding sites (Supplementary Fig. S3C) within the E-cadherin promoter.

Given that FOXA2 can activate the E-cadherin promoter/reporter in Snail1-expressing PANC-1 and MiaPaCa-2 cells (Fig. 5A), we asked whether FOXA2 is a dominant activating factor capable of overcoming E-cadherin-suppressing signals. Using the TGF β 1-responsive PANC-1 cells, we showed that both ectopic Snail1 overexpression and TGF β 1 treatment were able to repress wild-type E-cadherin promoter/reporter activity compared with control (Fig. 5B), as

expected. However, neither Snail1 nor TGFβ1 prevented FOXA2 activation of the E-cadherin promoter (Fig. 5B). In fact, FOXA2 activated the promoter to a level comparable to that when FOXA2 was expressed alone. These results indicate that downregulation of FOXA2 is important for Snail1 or TGFβ to effectively repress the E-cadherin promoter.

FOXA1/2 are potent activators of endogenous E-cadherin.
To test whether FOXA2 can activate the endogenous E-cadherin gene in the presence of other repressive mechanisms, a retrovirus-based FOXA2-IRES-enhanced

green fluorescent protein (EGFP) construct or an IRES-EGFP construct (as control) was stably expressed in PANC-1 cells. GFP⁺ cells were identified and enriched by FACS. Compared with control GFP⁺ cells, E-cadherin expression was strongly elevated in FOXA2-GFP⁺ cells, whereas vimentin expression was suppressed (Fig. 5C), consistent with FOXA2 promoting an epithelial character despite Snail1 being highly expressed in parental PANC-1 cells (Fig. 2B) and remaining unchanged in FOXA2-overexpressing cells (Fig. 5C).

We then tested whether FOXA1/2 overexpression can reactivate endogenous E-cadherin in E-cadherin-negative

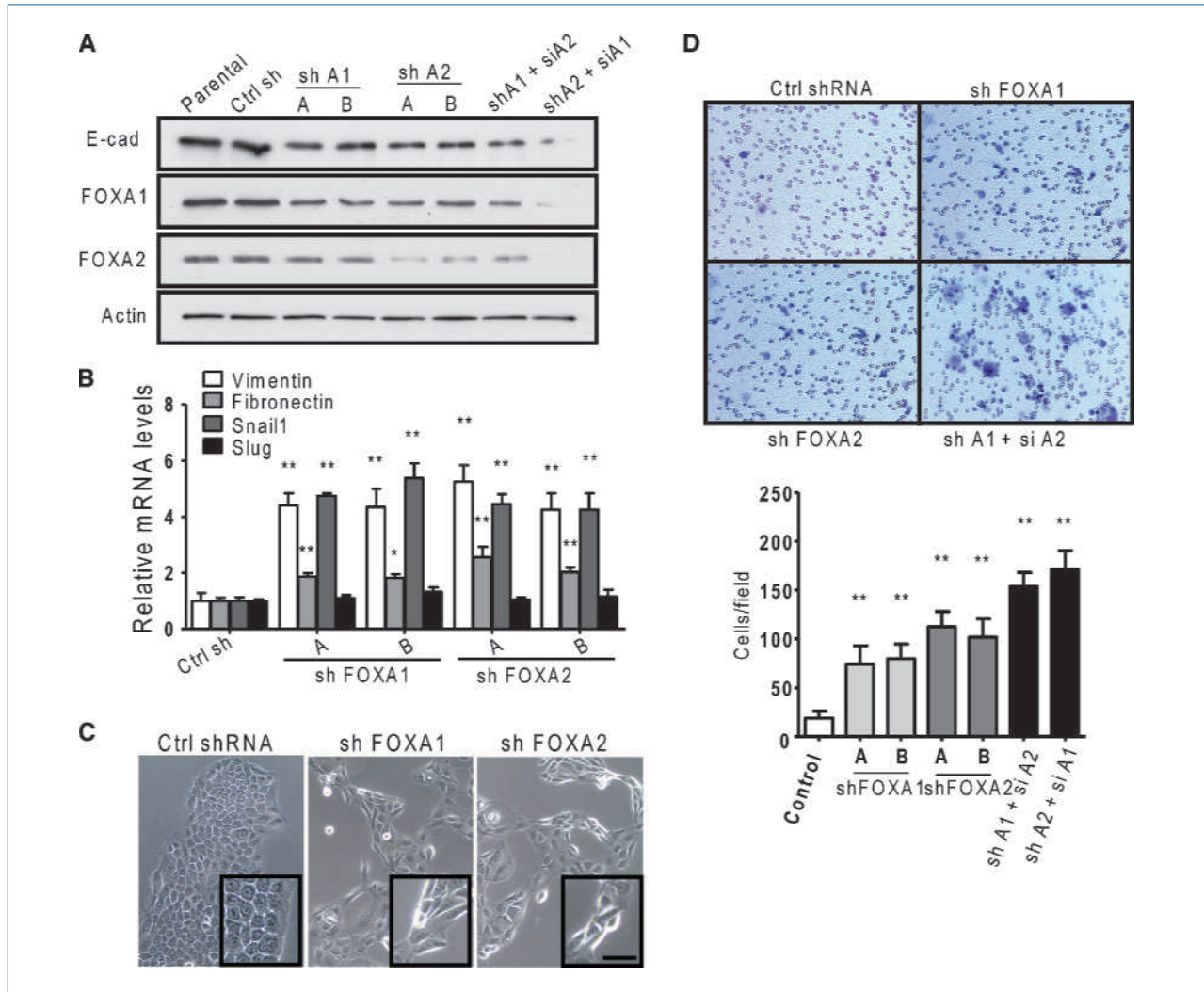


Figure 4. Persistent inhibition of FOXA1/2 induces EMT in PDA cells. BxPC3 cells with stably integrated shRNA constructs targeting either FOXA1 (shFOXA1) or FOXA2 (shFOXA2) were established by puromycin selection. Two independent shRNA constructs (designated A and B) were used for each gene. Cells with stable transfection of an empty pLKO.1 vector served as control. To analyze the combined effects of simultaneous FOXA1 and FOXA2 knockdown, siRNA targeting either FOXA2 or FOXA1 was transiently introduced into stable shFOXA1 or shFOXA2 BxPC3 cells, labeled shA1 + siA2 or shA2 + siA1, respectively. A, expression of FOXA1/2 and E-cadherin was examined by Western blot analysis in FOXA1/2-knockdown cells. When siRNAs were used, lysates were collected 48 h after siRNA transfection. Data shown are representative of three independent experiments. B, silencing of FOXA1/2 results in elevated mesenchymal markers analyzed by quantitative RT-PCR. Columns, mean from three independent experiments; bars, SEM. C, phase-contrast microscopy of control, shFOXA1, and shFOXA2 BxPC3 cells. Cells have increased cell spreading and a fibroblast-like shape in shFOXA1 and shFOXA2 BxPC3 cells. Bar, 100 μm (main pictures) and 50 μm (insets). D, silencing of FOXA1/2 results in increased cell migration in Transwell migration assays. Cells that migrated from serum-free to serum-containing medium within 6 h were stained with crystal violet (micrographs) and counted (bar graphs). Quantitation is of five random fields from each membrane and averaged from triplicates of three independent experiments. Bars, SEM. *, $P < 0.05$; **, $P < 0.01$.

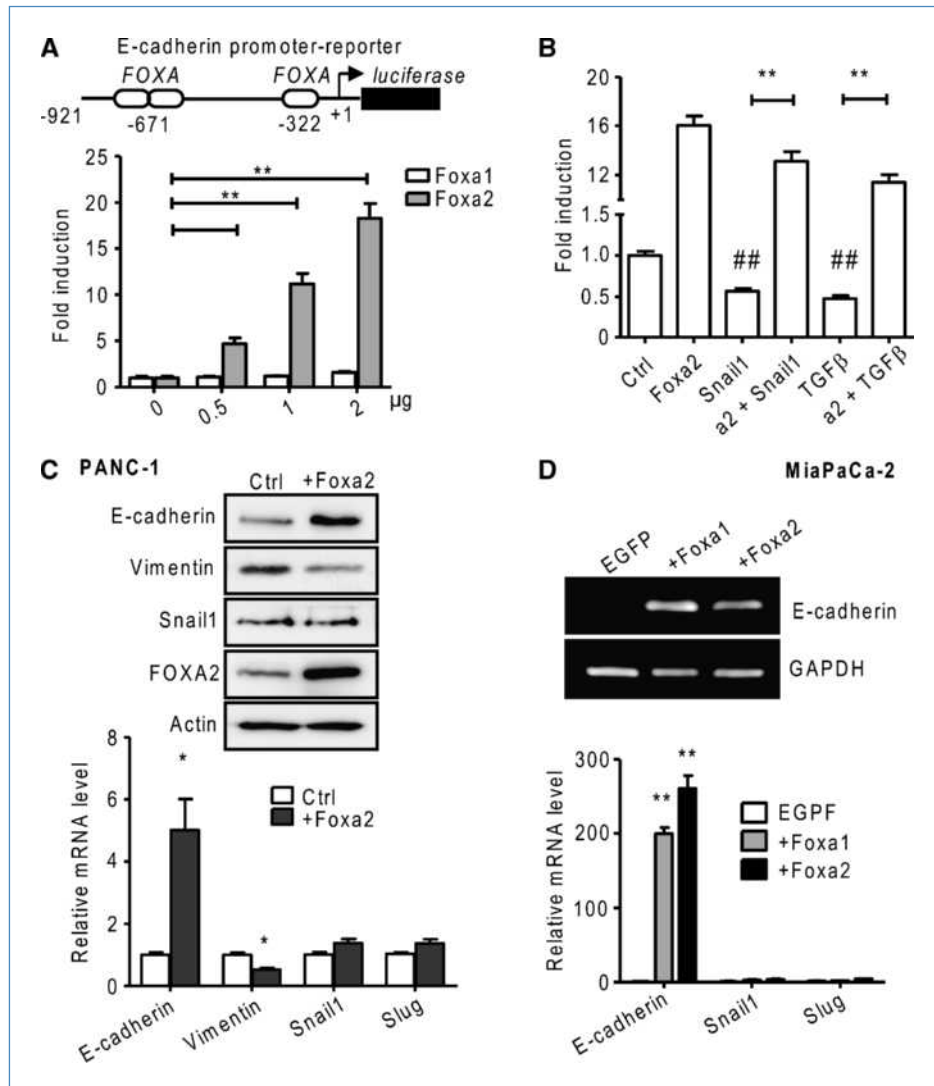


Figure 5. FOXA1/2 proteins are potent activators of *E-cadherin* in PDA cells. **A**, FOXA2 activates *E-cadherin* promoter/reporter. The schematic of the *E-cadherin* promoter fragment from -921 to +47 bp relative to the transcription start site is depicted with three FOXA binding sites. PANC-1 cells were cotransfected with an expression vector encoding EGFP, Foxa1, or Foxa2 with a wild-type -921 bp *E-cadherin* promoter/reporter. Fold induction is calculated relative to cotransfection with control EGFP vector. **B**, FOXA2 overcomes suppression of *E-cadherin* promoter mediated by Snail1 or TGF β . In PANC-1 cells, pGS-Snail1 (2 μ g) was cotransfected with an EGFP or Foxa2 expression vector (2 μ g). Twenty-four hours after transfection, cells were treated with 5 ng/mL TGF β , as indicated. Promoter activity was determined by dual-luciferase assay 48 h after transfection. Luciferase assays were done in triplicate and data were summarized from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ##, $P < 0.01$, in comparison with control. **C**, ectopic expression of Foxa2 enhances endogenous *E-cadherin* expression. A retrovirus-based Foxa2-IRES-GFP or IRES-GFP (control) construct was used to infect PANC-1 cells. GFP⁺ cells were enriched by FACS. Expression of *E-cadherin*, vimentin, Snail1, and FOXA2 was examined by Western blot (top) and quantitative RT-PCR (bottom) from GFP⁺ PANC-1 cells. **D**, ectopic expression of Foxa1/2 induces *E-cadherin* transcription in MiaPaCa-2 cells. MiaPaCa-2 cells were transiently transfected with an expression vector encoding EGFP, Foxa1, or Foxa2. *E-cadherin* mRNA was examined by semiquantitative RT-PCR (top) and by quantitative RT-PCR along with *Snail1* and *Slug* (bottom) 48 h after transfection. Representative results from three independent experiments are shown for C and D. Means \pm SEM are shown for quantitative RT-PCR from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

MiaPaCa-2 cells. MiaPaCa-2 cells express Snail1 (4) and have an *E-cadherin* promoter reported to be silenced by hypermethylation (29, 30) and an undetectable FOXA1/2 expression (Fig. 2). Given these multiple modes of *E-cadherin* repression, it was surprising that transient expression of either FOXA1 or FOXA2 elevated *E-cadherin* transcript levels, which were undetectable in parental and vector control cells,

to an easily detectable level (Fig. 5D), with a quantitative increase of >200-fold. Neither Snail1 nor Slug expression was repressed in these experiments (Fig. 5D). Although both promoter/reporter experiments (Fig. 5B) and ectopic FOXA2 overexpression in PANC-1 cells (Fig. 5C) showed that FOXA1/2 could counteract Snail1-mediated repression, it was unexpected that it could overcome promoter

hypermethylation. It should be noted that E-cadherin protein remained undetectable by Western blot with transient FOXA1 or FOXA2 expression (data not shown). It is possible that even with the dramatic elevation induced by FOXA1/2 factors, E-cadherin transcripts remain too low (~1% of BxPC3 levels, when adjusted for transfection efficiency) to lead to high levels of E-cadherin protein.

FOXA2 induction is responsible for demethylation-mediated E-cadherin reactivation. Hypermethylation of the *E-cadherin* promoter is thought to represent a silenced *E-cadherin* locus because it introduces an insurmountable structural impediment to transcription. Overexpression of FOXA1/2 proteins in MiaPaCa-2 cells, which contain mostly methylated *E-cadherin* promoter (ref. 30; Supplementary Fig. S4A), reactivated E-cadherin expression, showing that the promoter in these cells was amenable to transactivation. With this surprising result, we questioned whether promoter hypermethylation was demonstrably involved in E-cadherin silencing in MiaPaCa-2 cells. To test this, we reversed DNA methylation by treating cells with 5'-aza-dC (ref. 31; Supplementary Fig. S4A) and quantitated E-cadherin expression by quantitative RT-PCR. Indeed, *E-cadherin* transcripts were elevated by >20-fold after treatment (Fig. 6A), consistent with silencing by hypermethylation. However, we found that *FOXA2* transcripts were simultaneously elevated by ~10-fold after treatment (Fig. 6A), indicating that the *FOXA2* gene was also suppressed by methylation. A corresponding enhancement of E-cadherin and FOXA2 protein expression was found by FACS analysis (Fig. 6B). In contrast, no change was observed for FOXA1 (Fig. 6A; Supplementary Fig. S4B). Taken together, these results suggest the possibility that restoration of *E-cadherin* expression after demethylation may be indirect, through reactivation of FOXA2 expression.

To test this possibility, synthetic siRNAs targeting *FOXA2* were transfected into MiaPaCa-2 cells with or without 5'-aza-dC treatment. When FOXA2 expression was inhibited by either of the two *FOXA2* siRNAs, reexpression of *E-cadherin* mRNA was also inhibited by ~80% compared with control siRNA (Fig. 6C). This inhibition was confirmed at the protein level by FACS analysis (Fig. 6D). Thus, we conclude that, in MiaPaCa-2 cells, an indirect methylation-dependent regulatory mechanism controls E-cadherin expression through methylation-mediated suppression of *FOXA2*.

Discussion

Metastasis causes >90% of cancer deaths. The EMT is considered a prerequisite to metastasis for most carcinomas, allowing cancer cells to disassociate from the primary tumor and enhancing cell motility. Early observations showing that gain-of-function signals, such as oncogene activation and growth factor responses, are sufficient to induce EMT have led to the extensive study and identification of several EMT-promoting pathways, while relatively little attention has been paid to EMT-suppressing pathways. In this study, we show for the first time that inhibition of FOXA1/2 transcription factors is an integral part of the EMT program. Suppression of endogenous FOXA1/2 is sufficient to induce EMT,

and sustained FOXA1/2 expression can inhibit EMT induced by a variety of pathways.

Based on our findings from *in vivo* human PDA specimens and *in vitro* studies in PDA cell lines, we propose a model where FOXA1/2 factors are constitutively expressed in normal pancreatic epithelia, PanINs, and well-differentiated PDA, where they maintain optimal E-cadherin levels, thus suppressing EMT. As the cancer progresses, FOXA1/2 factors are suppressed by EMT-inducing signals (e.g., TGF β and DNA methylation), leading to downregulation of E-cadherin and possibly other epithelial markers. This model is consistent with previous observations of FOXA1/2 in breast cancer (17, 18, 22) and their functions during development (7). Concurrent with FOXA1/2 repression in PDA cells, E-cadherin repressors, such as Snail, are activated, further contributing to E-cadherin silencing and EMT induction. This model strongly suggests that the balance between FOXA factors and Snail repressors is critical. This idea is bolstered by our promoter/reporter studies in PANC-1 cells (Fig. 5B), which express both endogenous FOXA1/2 and Snail1 proteins yet are still susceptible to both Snail1 repression and FOXA2 activation of the E-cadherin promoter.

Similarly, the observed antagonism between FOX factors themselves may require their relative expression to be appropriately balanced as well. For instance, FOXC2 was shown to induce EMT and suppress E-cadherin expression (32), which could be achieved by antagonizing FOXA transactivation. Also, FOXA1 interferes with FOXA2-mediated transactivation as a result of its inferior transactivation activity (28). Although no activation of the E-cadherin promoter/reporter was observed with FOXA1 overexpression, coexpression of FOXA1 with FOXA2 dampened the degree of transactivation observed with FOXA2 overexpression alone by about 30% to 40% (data not shown), supporting the possibility of antagonism. However, because knockdown of endogenous FOXA1 in FOXA2-expressing lines consistently reduces E-cadherin expression (Figs. 3 and 4), we conclude that FOXA1 plays an overall positive role in endogenous *E-cadherin* gene expression, possibly by interacting with distal regulatory elements.

Another important mechanism implicated in E-cadherin regulation is DNA methylation-mediated gene silencing, conferred by establishment of repressive chromatin complexes composed of methyl CpG binding proteins and histone deacetylases that mediate chromatin condensation (33). Nevertheless, methylated promoters can still be activated by several transcriptional activators (34, 35) that compete with repressive chromatin complexes. Moreover, FOXA1 has been shown as a "pioneer factor" of chromatin remodeling that can decondense compacted chromatin through its COOH-terminal domain to facilitate further transcription (36, 37). We hypothesize that abundantly expressed FOXA proteins can activate a methylated E-cadherin promoter (Fig. 5D) due to their dual functions as potent trans-activators and chromatin remodeling factors.

Furthermore, we found that the *FOXA2* gene, but not the *FOXA1* gene, was regulated by methylation in an undifferentiated PDA cell line. Whether this is by direct methylation of

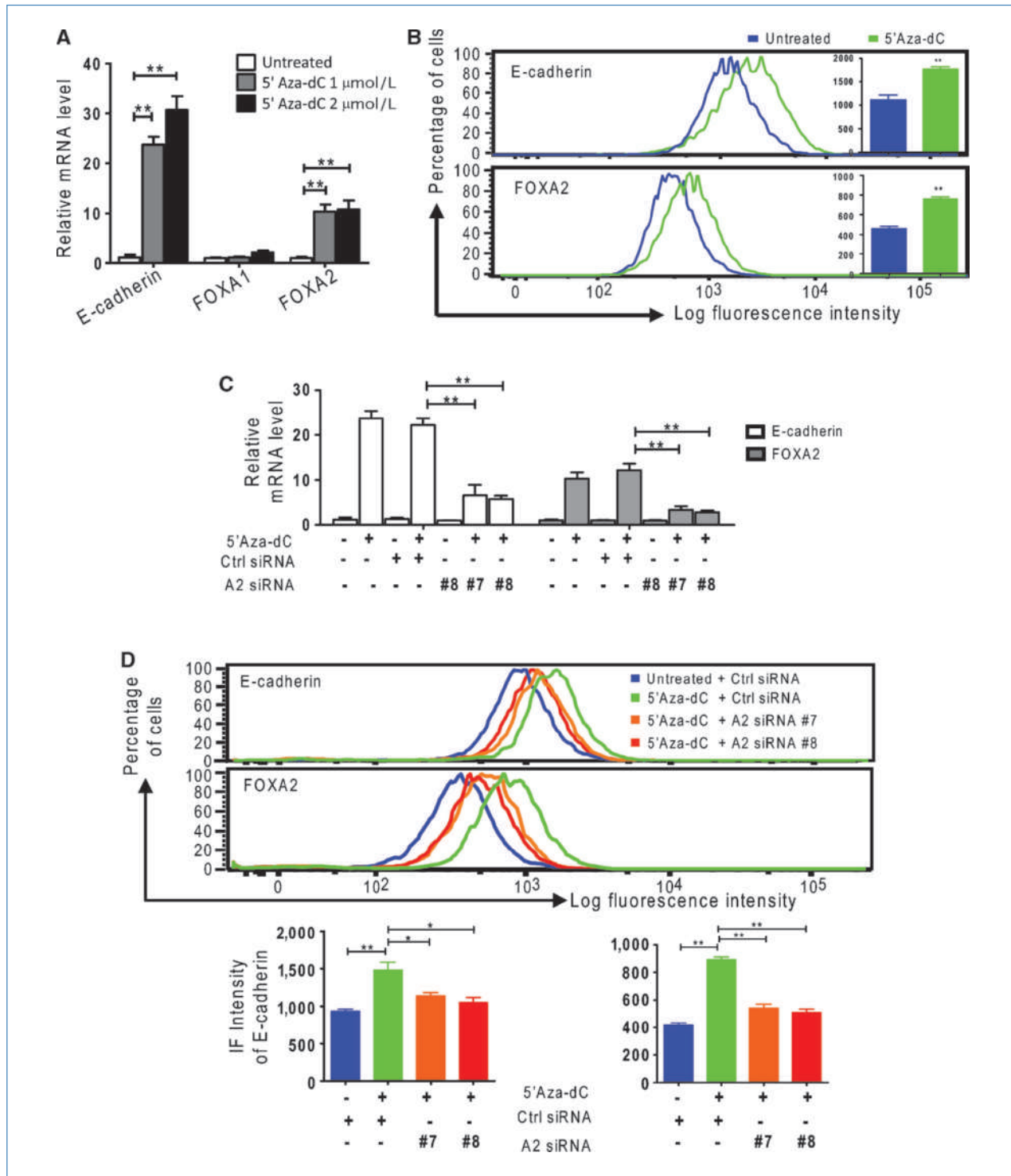


Figure 6. FOXA2 is required for demethylation-mediated *E-cadherin* reactivation. A and B, 5'-aza-dC treatment induces *E-cadherin* and FOXA2 expression. MiaPaCa-2 cells were treated with 5'-aza-dC for 5 d at the indicated doses. Expression of *E-cadherin*, FOXA1, and FOXA2 was examined by quantitative RT-PCR (A) and flow cytometry (B). C and D, 5'-aza-dC-induced *E-cadherin* expression is dependent on FOXA2 elevation. MiaPaCa-2 cells were treated with 1 μ mol/L 5'-aza-dC for 5 d. On the 3rd day, two different synthetic siRNAs (#7 and #8) against FOXA2 were independently transfected into 5'-aza-dC-treated or untreated MiaPaCa-2 cells. Expression of *E-cadherin* and FOXA2 was examined by quantitative RT-PCR (C) and flow cytometric analysis (D). For quantitative RT-PCR analysis, means \pm SEM from three independent experiments are shown. For FACS analysis, representative data from at least three independent experiments are shown. Only four data lines are shown for simplicity (representative complete data set is presented in Supplementary Fig. S4). Bar graph summarizes at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

the FOXA2 promoter or by methylation of upstream regulators, or both, is unknown. Two large CpG islands are predicted with heavily clustered CpG sites within a 2,100-bp 5' regulatory region of the *FOXA2* gene, using CpG island prediction softwares (cpgislands and cpgplot), making *FOXA2* a candidate for silencing by direct hypermethylation. Given the recent reports on methylation of E-cadherin promoter in less than 2% of PDA patient samples (30, 38), we propose that loss of E-cadherin expression may be strongly influenced by methylation-mediated silencing of the *FOXA2* gene, independent of direct methylation of the *E-cadherin* gene itself.

Besides the regulation of FOXA1/2 by EMT-inducing paracrine signals or DNA methylation, FOXA1/2 proteins frequently localized to the cytoplasm of undifferentiated cancer cells, suggesting an alternative mechanism of inhibiting their transcription activity. In an insulinoma cell line, cytoplasmic translocation of FOXA proteins was induced by AKT phosphorylation of the Fkhd domain (39) in a manner analogous to FOXO (40). However, such translocation of FOXA1 or FOXA2 was not found *in vivo* in response to phosphoinositide 3-kinase (PI3K)/AKT activation (7). In several PDA cell lines, we observed FOXA1 and FOXA2 in both the nucleus and the cytoplasm, but neither activation of AKT by growth factors nor pharmacologic inhibition of PI3K or AKT altered this distribution (data not shown). Nevertheless, we speculate that signaling pathways controlling nuclear-to-cytoplasmic shuttling of FOXA1/2 are likely to be important regulators of EMT in PDA.

References

- Bailey JM, Singh PK, Hollingsworth MA. Cancer metastasis facilitated by developmental pathways: Sonic hedgehog, Notch, and bone morphogenic proteins. *J Cell Biochem* 2007;102:829–39.
- Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2002;2:442–54.
- Yang J, Mani SA, Donaher JL, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* 2004;117:927–39.
- Cano A, Perez-Moreno MA, Rodrigo I, et al. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* 2000;2:76–83.
- Nieto MA, Sargent MG, Wilkinson DG, Cooke J. Control of cell behavior during vertebrate development by Slug, a zinc finger gene. *Science* 1994;264:835–9.
- Comijn J, Bex G, Vermassen P, et al. The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. *Mol Cell* 2001;7:1267–78.
- Friedman JR, Kaestner KH. The Foxa family of transcription factors in development and metabolism. *Cell Mol Life Sci* 2006;63:2317–28.
- Shih DQ, Navas MA, Kuwajima S, Duncan SA, Stoffel M. Impaired glucose homeostasis and neonatal mortality in hepatocyte nuclear factor 3 α -deficient mice. *Proc Natl Acad Sci U S A* 1999;96:10152–7.
- Kaestner KH, Katz J, Liu Y, Drucker DJ, Schutz G. Inactivation of the winged helix transcription factor HNF3 α affects glucose homeostasis and islet glucagon gene expression *in vivo*. *Genes Dev* 1999;13:495–504.
- Weinstein DC, Ruiz i Altaba A, Chen WS, et al. The winged-helix transcription factor HNF-3 β is required for notochord development in the mouse embryo. *Cell* 1994;78:575–88.
- Ang SL, Rossant J. HNF-3 β is essential for node and notochord formation in mouse development. *Cell* 1994;78:561–74.
- Lee CS, Sund NJ, Behr R, Herrera PL, Kaestner KH. Foxa2 is required for the differentiation of pancreatic α -cells. *Dev Biol* 2005;278:484–95.
- Lee CS, Friedman JR, Fulmer JT, Kaestner KH. The initiation of liver development is dependent on Foxa transcription factors. *Nature* 2005;435:944–7.
- Wan H, Kaestner KH, Ang SL, et al. Foxa2 regulates alveolarization and goblet cell hyperplasia. *Development* 2004;131:953–64.
- Gao N, Ishii K, Mirosevich J, et al. Forkhead box A1 regulates prostate ductal morphogenesis and promotes epithelial cell maturation. *Development* 2005;132:3431–43.
- Janni M, Nan G, Aparna G, Scott BS, Richard J, Robert JM. Expression and role of Foxa proteins in prostate cancer. *Prostate* 2006;66:1013–28.
- Thorat MA, Marchio C, Morimiya A, et al. Forkhead box A1 expression in breast cancer is associated with luminal subtype and good prognosis. *J Clin Pathol* 2008;61:327–32.
- Wolf I, Bose S, Williamson EA, Miller CW, Karlan BY, Koeffler HP. FOXA1: growth inhibitor and a favorable prognostic factor in human breast cancer. *Int J Cancer* 2007;120:1013–22.
- Lin L, Miller CT, Contreras JI, et al. The hepatocyte nuclear factor 3 α gene, HNF3 α (FOXA1), on chromosome band 14q13 is amplified and overexpressed in esophageal and lung adenocarcinomas. *Cancer Res* 2002;62:5273–9.
- Wu KL, Gannon M, Peshavaria M, et al. Hepatocyte nuclear factor 3 β is involved in pancreatic β -cell-specific transcription of the *pdx-1* gene. *Mol Cell Biol* 1997;17:6002–13.
- Besnard V, Wert SE, Hull WM, Whitsett JA. Immunohistochemical localization of Foxa1 and Foxa2 in mouse embryos and adult tissues. *Gene Expr Patterns* 2004;5:193–208.
- Liu YN, Lee WW, Wang CY, Chao TH, Chen Y, Chen JH. Regulatory mechanisms controlling human E-cadherin gene expression. *Oncogene* 2005;24:8277–90.

In summary, we have shown a consistent inhibition of FOXA1 and FOXA2 during EMT both *in vivo* and *in vitro* by diverse signals. Suppression of endogenous FOXA1/2 alone is sufficient to induce EMT in PDA cell lines and is required for E-cadherin silencing previously attributed directly to promoter hypermethylation. All together, these data lead us to conclude that loss of FOXA1/2 is an essential step in the EMT program during pancreatic cancer progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Drs. Robert Matusik (Vanderbilt University) and Holly Colognato (Stony Brook University) for plasmid gifts; Dr. Klaus Kaestner (University of Pennsylvania) for helpful advice; Dr. Jian Cao, Antoine Dufour, and Dr. Xing Chang (Yale University) for technical advice; and Dun Li for technical assistance.

Grant Support

National Cancer Institute grant R01CA100126 and the Knapp Chair for Pancreatic Cancer Research to H.C. Crawford and by grant P50CA095103 for the Vanderbilt University Medical Center GI Spore Tissue Core.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 08/10/2009; revised 11/17/2009; accepted 12/15/2009; published OnlineFirst 02/16/2010.

23. Battle E, Sancho E, Franci C, et al. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat Cell Biol* 2000;2:84–9.
24. Sipos B, Moser S, Kalthoff H, Torok V, Lohr M, Kloppel G. A comprehensive characterization of pancreatic ductal carcinoma cell lines: towards the establishment of an *in vitro* research platform. *Virchows Arch* 2003;442:444–52.
25. Horiguchi K, Shirakihara T, Nakano A, Imamura T, Miyazono K, Saitoh M. Role of Ras signaling in the induction of Snail by transforming growth factor- β . *J Biol Chem* 2009;284:245–53.
26. Ellenrieder V, Hendler SF, Boeck W, et al. Transforming growth factor β 1 treatment leads to an epithelial-mesenchymal transdifferentiation of pancreatic cancer cells requiring extracellular signal-regulated kinase 2 activation. *Cancer Res* 2001;61:4222–8.
27. Hirota M, Egami H, Corra S, et al. Production of scatter factor-like activity by a nitrosamine-induced pancreatic cancer cell line. *Carcinogenesis* 1993;14:259–64.
28. Duncan SA, Navas MA, Dufort D, Rossant J, Stoffel M. Regulation of a Transcription Factor Network Required for Differentiation and Metabolism. *Science* 1998;281:692–5.
29. Ueki T, Toyota M, Sohn T, et al. Hypermethylation of multiple genes in pancreatic adenocarcinoma. *Cancer Res* 2000;60:1835–9.
30. Winter JM, Ting AH, Vilardell F, et al. Absence of E-cadherin expression distinguishes noncohesive from cohesive pancreatic cancer. *Clin Cancer Res* 2008;14:412–8.
31. Lombaerts M, van Wezel T, Philippo K, et al. E-cadherin transcriptional downregulation by promoter methylation but not mutation is related to epithelial-to-mesenchymal transition in breast cancer cell lines. *Br J Cancer* 2006;94:661–71.
32. Mani SA, Yang J, Brooks M, et al. Mesenchyme Forkhead 1 (FOXC2) plays a key role in metastasis and is associated with aggressive basal-like breast cancers. *Proc Natl Acad Sci U S A* 2007;104:10069–74.
33. Bienvenu T, Chelly J. Molecular genetics of Rett syndrome: when DNA methylation goes unrecognized. *Nat Rev Genet* 2006;7:415–26.
34. Niesen MI, Osborne AR, Yang H, et al. Activation of a methylated promoter mediated by a sequence-specific DNA-binding protein, RFX. *J Biol Chem* 2005;280:38914–22.
35. Curradi M, Izzo A, Badaracco G, Landsberger N. Molecular mechanisms of gene silencing mediated by DNA methylation. *Mol Cell Biol* 2002;22:3157–73.
36. Cirillo LA, Lin FR, Cuesta I, Friedman D, Jarnik M, Zaret KS. Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. *Mol Cell* 2002;9:279–89.
37. Carroll JS, Liu XS, Brodsky AS, et al. Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. *Cell* 2005;122:33–43.
38. Peng DF, Kanai Y, Sawada M, et al. DNA methylation of multiple tumor-related genes in association with overexpression of DNA methyltransferase 1 (DNMT1) during multistage carcinogenesis of the pancreas. *Carcinogenesis* 2006;27:1160–8.
39. Wolfrum C, Besser D, Luca E, Stoffel M. Insulin regulates the activity of forkhead transcription factor Hnf-3 β /Foxa-2 by Akt-mediated phosphorylation and nuclear/cytosolic localization. *Proc Natl Acad Sci U S A* 2003;100:11624–9.
40. Brunet A, Bonni A, Zigmond MJ, et al. Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. *Cell* 1999;96:857–68.