The Transcription Factor Sterile Alpha Motif (SAM) Pointed Domain-containing ETS Transcription Factor (SPDEF) Is Required for E-cadherin Expression in Prostate Cancer Cells*

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Background: SPDEF functions like a tumor metastasis suppressor. However, the underlying mechanism remains unclear.

Results: SPDEF is required for E-cadherin expression in prostate epithelial cells.

Conclusion: SPDEF directly modulates E-cadherin expression in prostate cancer cells.

Significance: The results provide a potential mechanism by which SPDEF suppresses prostate cancer progression and metastasis.

Loss of E-cadherin is one of the key steps in tumor progression. Our previous studies demonstrate that SAM pointed domain-containing ETS transcription factor (SPDEF) inhibited prostate cancer metastasis in vitro and in vivo. In the present study, we evaluated the relationship between SPDEF and E-cadherin expression in an effort to better understand the mechanism of action of SPDEF in prostate tumor cell invasion/migration. The results presented here demonstrate a correlation between expression of E-cadherin and SPDEF in prostate cancer cells. Additional data demonstrate that down-regulation of E-cadherin and SPDEF decreased E-cadherin expression in prostate cancer cells. Taken together, our data support the idea that SPDEF is required for E-cadherin expression in prostate cancer cells.

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The progression of a tumor to an invasive tumor is a major prerequisite to cancer metastasis that requires the movement of cells through the basement membrane and the surrounding tissue (1). These processes include the acquisition of the migratory, invasive, and metastatic properties, and invasion of the actin cytoskeleton, a loss of cell-cell adhesion along with a gain of cell-matrix adhesion, and concomitant formation of membrane protrusions required for invasive growth (2). The molecular and cellular processes underlying such cellular changes are still poorly understood, and the various migratory, invasive, and metastatic cellular processes and play an important role in proliferation, differentiation, development, apoptosis, migration, invasion, and angiogenesis (3). Many ETS factors are deregulated and are thought to be key players in cancer (4). ETS factors have been shown to play a role in the majority of prostate cancer patients (5). SPDEF was originally identified and defined as a prostate-derived ETS factor, present in normal prostate luminal cells (6). SPDEF is unique among ETS factors because its expression is highly restricted to the tissues with high epithelial content, namely epithelial cells of prostate, mammary gland, endometrium, ovary, salivary gland, and colon (7). Although expression of SPDEF in cancer tissues remains debated, it is abundantly clear that SPDEF suppresses tumor metastasis in vitro and in vivo (7–10). We are the first group to demonstrate that decreased SPDEF expression is associated with an increased Gleason score in clinical samples of prostate cancer (10). We also demonstrated that there is an inverse relationship between SPDEF expression and MMP9 expression in the clinical samples in tissue microarray having both normal and cancerous tumor samples of prostate cancer (10). Our results demonstrated...
ing the loss of SPDEF and aggressive prostate cancer have been confirmed by at least two other independent studies (11, 12); one follow-up study in fact suggests that loss of SPDEF could be a predictor not only of aggressive prostate cancer but also of prostate cancer-associated death (12). Taken together, these studies clearly provide compelling evidence of the association between loss of SPDEF and aggressive prostate cancer. Therefore, seeking an understanding of the mechanisms by which SPDEF regulates cancer progression in general and prostate cancer in particular is highly warranted.

E-cadherin belongs to the cadherin family of calcium-dependent adhesion molecules and is highly expressed in normal epithelial cells and well differentiated cancer cells, but its expression is largely reduced in undifferentiated cancers (13). E-cadherin plays an important role in the maintenance of the structural integrity of epithelial sheets (14) and is regulated at both the transcriptional and post-transcriptional levels (15). Loss of E-cadherin expression has been regarded as a central event in tumor metastasis, because loss of adhesion between tumor cells facilitates their ability to invade locally and to spread to distant organs (16, 17). Many studies have focused on the relationship between loss of E-cadherin expression and the invasive and metastatic process. Recent studies have demonstrated that the loss of E-cadherin expression is frequently associated with parameters of enhanced biological aggressiveness such as poor histological differentiation, increased malignancy, metastatic disease, and a poorer survival rate with prostate (18), breast (19), bladder (20), hepatocellular (23), pancreatic (24), esophageal (25), thyroid (26), head and neck (27), and other kinds of cancer cells. Experimental studies in vitro and in vivo have indicated that loss of E-cadherin may be a useful marker of tumor progression (29). The elucidation of the mechanisms that regulate E-cadherin expression is essential to our understanding of tumor progression.

Because loss of SPDEF and E-cadherin expression in prostate cancer progression in several studies as described above, we set out to determine whether the association between expression of SPDEF and E-cadherin in prostate cancer cells. We also show for the first time that stable forced expression of SPDEF in prostate cancer cells up-regulates E-cadherin expression, whereas knockdown of SPDEF down-regulates E-cadherin expression. Moreover, modulating E-cadherin expression had no effect on SPDEF levels, indicating that SPDEF is upstream of E-cadherin. Moreover, SPDEF and E-cadherin expression decreased cell migration and invasion. Finally, we show that siRNA-mediated knockdown of E-cadherin impaired the ability of SPDEF to modulate cell migration and invasion. Most importantly, we show that SPDEF binds to the E-cadherin locus, suggesting a direct role for SPDEF in the regulation of E-cadherin expression. Taken together, our results provide the first direct demonstration of regulation of E-cadherin expression and a critical role for E-cadherin in modulating the function of SPDEF with respect to cell migration and invasion in prostate cancer.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—The reagents and antibodies were as follows: β-tubulin, and E-cadherin (Cell Signaling, Danvers, MA); Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 594 goat anti-mouse IgG (Molecular Probes/Invitrogen); HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); and cell culture medium (Cellgro; Mediatech Inc., Manassas, VA). E-cadherin GFP plasmid and E-cadherin promoter luciferase reporter plasmid (pGL2Basic-EcadK1) were from Addgene (Cambridge, MA). Oligonucleotide primers for PCR were purchased from Integrated DNA Technologies. Except where otherwise stated, all other chemicals were from Sigma-Aldrich.

**Constrictures and Cell Lines**—All cell lines (RWPE-1, PC3, LNCaP, LNCaP C4-2, and LNCaP C4-2B) were purchased from ATCC and maintained according to ATCC guidelines. Phoenix cells were grown in DMEM containing 10% fetal bovine serum. Cloning of SPDEF from human cDNA with an amino-terminal FLAG tag was performed using a eukaryotic vector pBABE and the bicistronic vector QCXIX (Clontech). Creation of stable SPDEF expressing cell lines was described previously. Construction of siRNA with E-cadherin GFP plasmid was performed using RNAiVenture Negative Control 1 (Qiagen) according to the manufacturer’s instructions. Transfection of siRNA was performed using HiPerFect reagent (Qiagen). The final concentration of the siRNAs was 30 nM.

**Western Analysis**—For Western blotting, protein extracts were made in ice-cold lysis buffer (20 mM Na2H2PO4, 250 mM NaCl, 1% Triton X-100, and 0.1% SDS). Western blot analysis was carried out as described previously (14).

**Immunofluorescence**—Staining was performed as described previously (9), and images were captured by LEICA DM RXA microscope using PL-AP 40×/1.25–0.75 oil objective over 15–20 high power fields.

**Total RNA Isolation and Real Time Quantitative PCR**—Total RNA was purified from homogenized cells using RNAeasy kit (Qiagen). One μg of total RNA was reverse transcribed using a cDNA synthesis kit (Bio-Rad), and quantitative PCR was performed as described previously (30). Expression was related to the control gene (GAPDH), which did not change under the experimental conditions studied.

**Chromatin Immunoprecipitation Assay**—ChIP was performed as described previously (30), except that anti-SPDEF
E-cadherin Expression Requires SPDEF

**TABLE 1**

| Gene name (human) | qRT-PCR primers (5' → 3') | Size |
|-------------------|---------------------------|------|
| Control           | TCACCTCGCTGCAAGCAGCGCC    | 294  |
| SPDEF             | AATGTGTCGAGGGAAAAATAGG    | 115  |
| Sense             | GGGTGTCGAGGGAAAAATAGG     |      |
| Antisense         | TGGTGGGGAGAGTTGTGGCT      |      |
| E-cadherin        | CGGAGACTACCTTCACTACAGC    |      |
| Sense             | AATGTGTCGAGGGAAAAATAGG    | 115  |
| Antisense         | TGGTGGGGAGAGTTGTGGCT      |      |

*Melting curve analysis was performed to assure that only PCR product was formed. Primers were designed to generate a PCR amplification product of 100–550 bp. Only primer pairs yielding unique amplification products without primer-dimer formation were subsequently used for real time PCR assays.*

**RESULTS**

**E-cadherin Expression Requires SPDEF**

In vitro scratch wound healing tests using SPSS software. The values are expressed as the means ± S.E., and *p* < 0.05 was considered statistically significant.

**Data Analysis**

The experiments presented in the figures are representative of three different experiments analyzed statistically by two-tailed Student's *t* tests. The values are expressed as mean ± S.E., and *p* < 0.05 was considered statistically significant.

**RESULTS**

**Loss of SPDEF Is Associated with Loss of E-cadherin in Prostate Cancer**

First, we investigated the correlation between SPDEF and E-cadherin mRNA expression levels in different prostate cancer cell lines by quantitative real time RT-PCR (qRT-PCR) on a panel of immortalized prostate cancer cell lines (LNCaP, LNCaP C4-2, LNCaP C4-2B, and PC3) with varying degrees of tumorigenic and metastatic potential to compare expression levels of SPDEF and E-cadherin with respect to non-tumorigenic human prostate epithelial cells, RWPE-1. The results of these studies show for the first time a direct correlation between expression of E-cadherin and SPDEF in four prostate cancer cell lines and one normal prostate cell line (Fig. 1, A and B). All the indicated cell lines showed significant (*p* < 0.05) decreases in E-cadherin, as well as SPDEF expression, when compared with normal prostate epithelial cells, RWPE-1. Importantly, the expression of E-cadherin in normal prostate epithelial cells (N-14; Santa Cruz Biotechnology) and anti-FLAG M2 (Sigma-Aldrich) antibodies were used. The sequence of the ChIP primers was as given in Table 1.

**Reporter Assay**

Relative luciferase activity was performed as described previously (31). In brief, the cell lysate was harvested 24 h post-transfection of E-cadherin reporter plasmid in stable pBABE Vec/pBABE SPDEF PC3 and Scr/SPDEF KD LNCaP cells, and luciferase activity was measured relative to total cell lysate protein for transfection efficiency.

**Cell Migration and Invasion Assay**

The experiment was performed as described previously (31). Confluent cultures in serum-free medium.

**Down-regulation of SPDEF Decreases E-cadherin Expression, but siRNA-mediated Knockdown of E-cadherin Does Not Effect on SPDEF Expression in LNCaP Cells**

To further examine the relationship between SPDEF and E-cadherin expression, we knocked down SPDEF in LNCaP cells using SPDEF-specific shRNA. Next we performed qRT-PCR and Western blot analysis of SPDEF and E-cadherin in these cells. The results of these studies, presented in Fig. 3A, show that shRNA-mediated knockdown of SPDEF in LNCaP cells significantly decreased E-cadherin mRNA expression (*p* < 0.05), as well as E-cadherin protein levels (Fig. 3B). These results further support the notion of modulation of E-cadherin by SPDEF.

To further test whether or not E-cadherin expression had any role in regulating SPDEF expression, we performed siRNA-mediated knockdown of E-cadherin in LNCaP cells and
evaluated expression of SPDEF in these cells. The results of these studies show that E-cadherin-specific siRNA significantly ($p < 0.05$) decreased E-cadherin expression. However, there was no significant change in SPDEF expression following E-cadherin knockdown (Fig. 3, C and D), suggesting a common underlying mechanism for the regulatory roles of SPDEF on
E-cadherin Expression Requires SPDEF

FIGURE 3. Down-regulation of SPDEF decreases E-cadherin expression. A and B, expression profiles of SPDEF and E-cadherin mRNA (A) and protein (B) measured by qRT-PCR and Western blot in SPDEF-specific shRNA-mediated knockdown into LNCaP cells. C and D, mRNA (C) and Western blot (D) results showing the significant changes of expression levels of E-cadherin in siRNA-mediated (30 nM) knockdown in PC3 cells, but no significant changes of expression level of SPDEF. Shown are densitometric analyses of three independent experiments using ImageJ software. *, p < 0.05.

E-cadherin expression in different prostate cancer cell lines. To further confirm these findings, we performed additional studies using overexpression of E-cadherin in PC3 cells, as well as knockdown of E-cadherin in pBABE SPDEF PC3 cells. These results from these studies (Fig. 4) confirmed that modulation of E-cadherin had no significant effect on SPDEF expression. Taken together with other studies, our results demonstrate that SPDEF regulates expression of E-cadherin, whereas E-cadherin does not regulate SPDEF expression.

In Silico Analysis of the Promoter Region of E-cadherin—The ETS family of transcription factors bind to a core GGAT sequence present upstream of target genes through its interaction with ETS core binding sites present upstream of target genes. SPDEF directly regulated E-cadherin promoter activity. Thus, SPDEF physically associates with the E-cadherin proximal promoter in vivo and could therefore function as a direct transcriptional inducer of E-cadherin.

SPDEF Delayed the Migration and Invasion of Prostate Cancer PC3 and LNCaP Cells by Modulating E-cadherin Expression—Our previous studies have shown that SPDEF stable expression suppresses cell migration. Therefore, we set to further test the role of E-cadherin in mediating the effects of SPDEF on cell migration. For these studies, the cells were seeded 24 h before the wound was generated, and the uncovered area of wound was subsequently measured at 0, 24, and 48 h. The results showed that the migration rate of SPDEF-transfected (Fig. 6A, left panel, and B, top panel) or E-cadherin-transfected (Fig. 6C, left panel, and D, top panel) PC3 cells decreased as compared with respective control cells at the same time point. Cells expressing SPDEF or E-cadherin failed to close the wound scratch at 48 h after wounding, whereas control cells were able to move to an almost complete closure. These results suggest that up-regulation of SPDEF/E-cadherin decreased cell migration. In SPDEF- and E-cadherin-transfected PC3 cells, cell-free area was increased by more than 6-fold compared with respective Vec-treated cells at 48 h (p < 0.005). During the incubation
period, the cells moved forward and closed the gap independently of cell division. There was no significant increase in cell growth within 48 h under the same treatment conditions as revealed by cell proliferation assay (data not shown). Similar effects of SPDEF and E-cadherin expression were observed on cell invasion (Fig. 6, right panels, and B and D, bottom panels).

To further confirm the role of SPDEF on cell migration and invasion in other prostate cancer cell lines, we performed in vitro cell migration and invasion assay using LNCaP SPDEF KD cells. The closure of the wounded gap was significantly accelerated in LNCaP SPDEF KD monolayer cells compared with respective controls (LNCaP Scr) at 48 h (Fig. 7, A and B, left panels). Cell invasion assay confirmed a similar migratory prototype (Fig. 7, A and B, right panels) as in cell migration assay.

In complementary experiments, we performed siRNA-mediated E-cadherin knockdown of pBABE SPDEF PC3 and LNCaP cells. Interestingly, consistent with the above observation, our live imaging results showed that E-cadherin knockdown into both cell lines closed the wound by 72 h ahead as compared with its respective control, which failed to close the wound by the end of the 48-h experimental period (Fig. 8, A and C, left panels, and B and D, top panels). The uncovered area at the indicated time points relative to control at 0 h was determined by ImageJ software. **, p < 0.005.
mined (Fig. 8, B and D, top panels). The results demonstrated that the rates of cell migration of E-cadherin knockdown in pBABE SPDEF PC3 and LNCaP cells were significantly \( p < 0.05 \) higher than their respective control cells (Fig. 8, B and D, top panels). We also found that the numbers of invasive cells in siRNA-mediated E-cadherin knockdown pBABE SPDEF PC3 and LNCaP cells were 3.5- and 6.0-fold higher than their respective controls (Fig. 8, A and C, right panels, and B and D, bottom panels). Transwell Matrigel invasion assays were performed in LNCaP cells. The cells were counted from five random microscopic fields per insert in triplicate. The migrated cell numbers were normalized to that of the control group. **, \( p < 0.005 \).

![WITHDRAWN Image](http://www.jbc.org/)

**FIGURE 6.** Forced expression of SPDEF and/or E-cadherin impairs cell migration and invasion of prostate cancer cells. Representative time-lapsed images of wounded cultures were captured at 0, 24, and 48 h after wounding in serum-free medium using pBABE Vec (A, left panel, and B, upper panel) or pBABE SPDEF (C, left panel, and D, upper panel) pcDNA 3.1 Vec or pcDNA 3.1 E-cadherin transfected PC3 cells. Yellow dotted lines represent the scratch gap at the time of wounding. Total magnification, \( 4 \times \). Transwell Matrigel invasion assays were performed in pBABE Vec (A, right panel, and B, lower panel) or pBABE SPDEF (C, right panel, and D, lower panel) pcDNA 3.1 Vec or pcDNA 3.1 E-cadherin transfected PC3 cells. The distance to be covered at 0, 24, and 48 h was normalized to that of the control group. **, \( p < 0.05 \).

**FIGURE 7.** SPDEF knockdown increases cell migration and invasion of prostate cancer cells. A, left panel, representative time-lapsed phage images of wounded cultures in LNCaP cells. Total magnification, \( 4 \times \). B, left panel, percentages of wound uncovered at all time points under each condition normalized to the initial at 0 h. A and B, right panels, Transwell Matrigel invasion assays were performed in LNCaP cells. The number of cells that migrated across the membranes per imaging field was counted from five random microscopic fields per insert in triplicate. **, \( p < 0.005 \).

The study we have discovered that, in prostate cancer, the loss of SPDEF is associated with loss of E-cadherin. We also show that SPDEF-mediated suppression of cell migration and invasion is dependent in part on E-cadherin expression. Using complementary experiments, we demonstrate that SPDEF is a transcriptional regulator of E-cadherin. To our knowledge, this is the first study to demonstrate that SPDEF suppresses tumor metastasis, in part, by modulating E-cadherin expression in cancer cells. Given the important role of E-cadherin in tumor metastasis (35), coupled with the regulation of E-cadherin by SPDEF as shown here, SPDEF expression may play a critical role in cancer progression in general and in prostate cancer progression in particular.

The results of our studies show that decreased expression of SPDEF is associated with decreased E-cadherin levels in prostate cancer cells. Loss of E-cadherin has been associated with cancer progression in several tissues including prostate cancer (18–21, 36). It is important to mention that current markers in prostate cancer fail to distinguish between indolent disease and an aggressive and often lethal cancer. There is at present an unmet need for new markers that can separate indolent prostate cancer from lethal disease. Our results, coupled with previous findings by others and us (10–12) that loss of SPDEF...
might serve as a prognostic indicator of aggressive prostate cancer, offer a potential use for SPDEF and E-cadherin as markers for distinguishing indolent prostate cancer from aggressive disease.

Moreover, these results, of decreased SPDEF expression in invasive prostate cancer cells, offer a potential role for SPDEF and E-cadherin as markers for distinguishing indolent prostate cancer from aggressive disease.

The precise mechanisms responsible for E-cadherin inactivation in prostate cancer cells are not completely understood. Alterations seen in PC3 cells in three-dimensional culture in our previous studies (10). Thus, our results are consistent with regulation of metastasis by SPDEF-forced expression in aggressive prostate cancer cells.

The precise mechanisms responsible for E-cadherin inactivation in prostate cancer are not completely understood (45–50). Given that both EMT and anoikis resistance in prostate cancer cells (9). However, we observed an inverse correlation between SPDEF and Twist expression in prostate cancer cells, indicating a potential role for SPDEF and E-cadherin in prostate cancer cells. The movement of cells that characterizes tumor progression, when E-cadherin silencing causes loss of cell-cell adhesion and results in gain of the invasive phenotype (51). Our findings demonstrate that the critical requirement of SPDEF for E-cadherin expression is thus highly relevant to tumor progression. The movement of cells that characterizes this process is brought about by a switch in E-cadherin expression, which causes them to lose their ordered epithelial phenotype and become plastic. This is analogous to late stage tumorigenesis, when E-cadherin silencing causes loss of cell-cell adhesion and results in gain of the invasive phenotype (51). Thus, our results are consistent with regulation of metastasis by SPDEF. Re-expression of E-cadherin following SPDEF expression in prostate cancer cells may also explain phenotypic alterations seen in PC3 cells in three-dimensional culture in our previous studies (10).

The precise mechanisms responsible for E-cadherin inactivation in cancer cells are not completely understood. Alterations at the transcriptional level have been proposed to be one of the major mechanisms responsible for decreased expression of E-cadherin in several cancer types (45–47). Inactivation of cell adhesion in cancer progression has been linked to modulation of E-cadherin by multiple mechanisms, such as a gene...
mutation, promoter hypermethylated, chromatin rearrangement, post-translational truncation or modification, and the recently characterized transcriptional repression (45, 46, 48–50). Our results suggest that SPDEF serves as a transcriptional activator of E-cadherin. This finding was verified by qRT-PCR and immunoblotting on a series of immortalized prostate cancer cell lines that reflect many features of cancer cells in vivo (10). E-cadherin has a tissue-specific distribution in both mice and humans (39), implying the existence of a finely tuned regulatory mechanism for E-cadherin gene expression. Our in silico computational analysis of human E-cadherin promoter sequence revealed the presence of five specific SPDEF-binding (GGAT) sequences in the short stretch of 1.5 kb upstream of the transcription start site. Indeed, using E-cadherin promoter-driven luciferase assay, we demonstrate that SPDEF is required for E-cadherin promoter activity. Moreover, our results demonstrate that SPDEF occupies the E-cadherin promoter in ChIP analysis (Fig. 5B). These results further strengthen the role of SPDEF in transcriptional regulation of E-cadherin.

We observed that E-cadherin expression could reverse the migratory and invasive phenotype that is driven by loss of SPDEF in prostate cancer cells (Fig. 6). In complementary studies, we observed that suppression of E-cadherin prevents the effects of SPDEF on cell migration and invasion (Fig. 7A, bottom). Together, these results indicate that the affects of SPDEF are at least with respect to its role in modulating cell migration and invasion, in part mediated by E-cadherin. It is interesting to point out that SPDEF has been shown to regulate several genes that are associated with metastasis in various cancer cells (46–50). Our results suggest that SPDEF serves as a transcriptional repressor in general and prostate tumor metastasis suppressor in particular.

In summary, our studies highlight the role of SPDEF as a transcriptional regulator of E-cadherin and offer a new potential mechanism by which SPDEF could suppress cancer progression and metastasis. Thus, decreased expression of SPDEF and E-cadherin may serve not only as indicators of aggressive prostate cancer but may also serve as novel targets in management of aggressive prostate cancer and perhaps other malignancies. However, additional studies are necessary to cement these conclusions.

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