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Promoter Methylation Blocks *FES* Protein-tyrosine Kinase Gene Expression in Colorectal Cancer

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Abstract

The *FES* locus encodes a unique non-receptor protein-tyrosine kinase (FES) traditionally viewed as a proto-oncogene but more recently implicated as a tumor suppressor in colorectal cancer (CRC). Recent studies have demonstrated that while FES is expressed in normal colonic epithelium, expression is lost in tumor tissue and colorectal cancer cell lines, a finding common among tumor suppressors. Here we provide compelling evidence that promoter methylation is an important mechanism responsible for down-regulation of *FES* gene expression in colorectal cancer cells. Treatment with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine resulted in the expression of functional *FES* transcripts in all CRC cell lines examined, including Caco-2, COLO 320, DLD-1, HCT 116, SNU-1040, SW-480, and HT-29. Bisulfite sequencing of genomic DNA isolated from 5-aza-2′-deoxycytidine-treated HT-29 cells identified methylated CpG dinucleotides immediately upstream from the *FES* transcription initiation sites. In contrast, this region of the *FES* promoter was hypomethylated in genomic DNA from normal colonic epithelium. In addition, methylation completely blocked the activity of the *FES* promoter in reporter gene assays. Promoter methylation is a previously unrecognized mechanism by which *FES* expression is suppressed in CRC cell lines, and is consistent with a tumor suppressor role for *FES* in this tumor site despite its tyrosine kinase activity.

INTRODUCTION

The human *FES* locus encodes a 93 kDa protein-tyrosine kinase (FES) expressed in myeloid, vascular endothelial, neuronal, and epithelial cells (Haigh et al., 1996; Smithgall et al., 1998; Greer, 2002; Delfino et al., 2006b). The *FES* gene was first identified as the normal cellular homolog of transforming oncogenes found in avian and feline retroviruses (Smithgall et al., 1998; Greer, 2002). Unlike its transforming viral counterparts, which exhibit constitutive protein-tyrosine kinase activity, FES kinase activity is strictly regulated in mammalian cells (Greer et al., 1988; Feldman et al., 1989). However, ectopic over-expression of wild-type Fes or of activated Fes mutants causes oncogenic transformation of rodent fibroblasts as well as tissue hyperplasia and hemangioma formation in transgenic mice (Feldman et al., 1989; Greer et al., 1994; Cheng et al., 2001). These earlier findings led to the view that *FES* functions as a proto-oncogene. However, over-expression of wild-type Fes in K-562 myeloid leukemia cells suppresses cell growth and restores differentiation, implicating Fes as a potential suppressor of chronic myelogenous leukemia (Yu et al., 1989; Lionberger and Smithgall, 2000; Rogers et al., 2000).

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Recent studies have proposed a novel role for FES as a tumor suppressor in epithelial cells as well. Bardelli and colleagues discovered that *FES* was one of only seven genes exhibiting consistent colorectal cancer-associated kinase domain mutations following nucleotide sequence analysis of the tyrosine kinome of 182 colorectal cancers (Bardelli et al., 2003). While these mutations were initially predicted to be activating and contribute to tumorigenesis, subsequent studies have established that these mutations rendered FES either catalytically inactive or had no effect on kinase activity (Sangrar et al., 2005; Delfino et al., 2006b). Using a mouse breast epithelial cancer model system, Greer and colleagues determined that tumor onset occurred more rapidly in mice targeted with either null or kinase-inactivating *FES* mutations and that a *FES* transgene restored the kinetics of tumor onset in the *FES* null mice (Sangrar et al., 2005). Our group determined that re-expression of wild-type or activated Fes suppressed the growth of the Fes-negative HT-29 and HCT 116 colorectal cancer (CRC) cell lines in soft agar (Delfino et al., 2006b). Our study also showed that while FES was strongly expressed in normal colonic epithelial cells from CRC patient samples, expression was reduced or absent in 67% of colon tumor sections from the same group of individuals (Delfino et al., 2006b). Similarly, Fes protein expression was significantly reduced or absent in five of six CRC cell lines examined (Delfino et al., 2006b). Together, these results suggest that loss of FES expression is a common finding in colorectal cancer, an observation that fits with a tumor suppressor function for FES in this tumor site. However, the mechanisms responsible for FES protein loss in colonic epithelial cells are currently unknown.

Epigenetic silencing of tumor suppressor gene transcription, through DNA methylation and histone modifications, is well-recognized as a ‘third pathway’ in Knudson’s model of tumor suppressor inactivation in cancer (Kondo and Issa, 2004). DNA methylation events typically occur at carbon 5 of cytosine in CpG (5′-CG-3′) dinucleotide sequences, a reaction that is catalyzed by DNA (C5) methyltransferases. In normal cells, CpG islands (CpG-rich stretches of DNA approximately 1 kb in length) within a gene promoter are rarely methylated (Baylin and Ohm, 2006). However, when promoter CpG islands become hypermethylated, transcription of downstream genes is often compromised (Ushijima, 2005; Baylin and Ohm, 2006). In fact, methylation of a CpG island in a tumor suppressor gene promoter often leads to irreversible inhibition of expression (Jones and Takai, 2001; Christman, 2002; Jones and Baylin, 2002; Herman and Baylin, 2003).

In this study, we investigated promoter methylation as a possible mechanism responsible for the loss of *FES* gene expression associated with colorectal cancer. We first established that the absence of FES protein in CRC cell lines correlates with the loss of full-length *FES* transcripts. Computational analysis of the *FES* promoter region revealed the presence of a putative CpG island surrounding the transcription initiation sites. Subsequent 5-aza-2′-deoxycytidine demethylation experiments restored Fes gene and protein expression in all of CRC cell lines analyzed, and bisulfite sequencing experiments identified key methylated CpG dinucleotides within the *FES* promoter region that may be responsible for gene silencing. Finally, *in vitro* methylation completely blocked the activity of the *FES* promoter in reporter-gene assays, directly implicating methylation as a major mechanism suppressing *FES* expression in colorectal cancer.

MATERIALS AND METHODS

Cell culture

Cell lines were maintained at 37 °C in a 5% CO_2 humidified incubator. Caco-2 and 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen). COLO 320, DLD-1, SNU-1040, and SW-480 cells were grown in RPMI 1640 medium (Invitrogen). HCT 116 and HT-29 cells were grown in McCoy’s Modified 5A medium (Invitrogen). TF-1

and K-562 cell culture has been previously detailed (Cheng et al., 1999; Cheng et al., 2001). All cell culture media were supplemented with 10% fetal bovine serum (FBS) (Atlanta Biological) and Antibiotic/Antimycotic (Invitrogen). All cell lines were obtained from the American Type Culture Collection.

RT-PCR

Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) and treated with TURBO DNase (Ambion) to remove contaminating traces of DNA. Random decamer-primed cDNA was synthesized from 1 μg of total RNA using the RETROscript Kit (Ambion). For semiquantitative analysis, one-tenth of each RT reaction was used in a 50 μl PCR reaction. Amplification of Fes and GAPDH were performed as follows: 94 °C for 2 min; 35 or 40 cycles at 94 °C for 1 min, 59 °C or 65 °C for 30 sec, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. The primer pair used to amplify a 3′ portion of the *FES* transcript (280 bp PCR product from the kinase domain coding region) was 5′-GGACCTGGCTGCTCGGAACTG-3′ (sense) and 5′-CCTTCTCCACAAACTCCCGTGTC-3′ (antisense). The primer pair used to amplify a 5′ portion of the *FES* transcript (266 bp PCR product encompassing non-coding exon 1 through the FCH domain coding region in exon 2) was 5′-GAGGAGGAAGCGCGGAATCAG-3′ (sense) and 5′-CTCGAATTCTCACCCACTGTCCTGCAGGG-3′ (antisense). The GAPDH control was amplified using the primer pair 5′-CCCTTCATTGACCTCAACTACATGGT-3′ (sense) and 5′-GAGGGGCCATCCACAGTCTTCTG-3′ (antisense) to generate a 470 bp product. Aliquots (10 μl) of each reaction were run on 2% agarose gels and stained with ethidium bromide.

Southern Blot Analysis

Genomic DNA was isolated using the PureLink Genomic DNA Purification Kit (Invitrogen). Southern blot analysis was performed as previously described (Smithgall et al., 1991). Briefly, aliquots of genomic DNA (10 μg) were digested overnight with 300 U each of *Eco*RI and *Bam*HI at 37 °C. DNA fragments were resolved on an 0.8% agarose gel, denatured, and transferred to a nylon membrane by the capillary method in 20X SSC overnight. The DNA was subsequently crosslinked to the membrane by ultraviolet irradiation (Spectrolinker XL-1000; Stratgene). The membrane was then prehybridized for 2 hours at 42 °C in prehybridization buffer (50% formamide, 3X SSC, 10X Denhardt's solution, 2% SDS, and 40 μg/ml of heat-denatured herring sperm DNA). Hybridization was conducted overnight at 42 °C in hybridization buffer (50% formamide, 3X SSC, 1X Denhardt's solution, 5% dextran sulfate, 2% SDS, and 40 μg/ml of heat-denatured herring sperm DNA) containing 10^6 cpm/ml of each of two ^{32}P-labeled *FES*-specific probes described previously (Smithgall et al., 1991). After hybridization, the membrane was washed twice in 2X SSC/0.1% SDS at 42 °C for 15 min, followed by two 15 min washes in 0.2X SSC/0.1% SDS at 55 °C. Detection of *FES* sequences required autoradiography for five days using intensifying screens.

5-aza-2′-deoxycytidine (5-aza-2′-dC) treatment

Freshly plated cells were treated with 5-aza-2′-dC (Sigma) at a final concentration of 10–15 μM (Fang et al., 2003). The 5-aza-2′-dC treatments were renewed every 24 hours. Total RNA, protein, or genomic DNA was isolated from the treated cells for analysis of *FES* mRNA levels (4 days), FES protein levels (4 days), and *FES* promoter methylation status (8 days).

Immunoprecipitation, immunoblotting, and antibodies

Cultured cells were washed with PBS, harvested by centrifugation, and sonicated on ice in radioimmune precipitation assay (RIPA) buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 2 mM sodium orthovanadate, 25 mM sodium fluoride and Protease Inhibitor Cocktail Set III (EMD Biosciences)]. Fes immunoprecipitation assays were performed as described elsewhere (Delfino et al., 2006a). In brief, clarified protein lysates were incubated with a rabbit polyclonal antiserum raised against the Fes amino-terminal and SH2 domains (Haigh et al., 1996) for 1 h at 4 °C and subsequently incubated for an addition hour at 4 °C with 30 μl of protein G-Sepharose beads (AP Biotech; 1:1 w/v slurry). Following three washes with RIPA buffer, the protein complexes were eluted from the beads by heating in SDS sample buffer. Immunoprecipitates were subjected to immunoblot analysis with antibodies to FES (Santa Cruz Biotechnology, C-19; 1 μg/ml). Lysates were blotted with actin antibodies (Chemicon MAB1501; 1:1000 dilution) to control for equivalent amounts of input protein in each immunoprecipitation reaction.

Tissue Staining

Formalin-fixed, paraffin-embedded normal human colon surgical specimens (obtained from the University of Pittsburgh Health Sciences Tissue Bank) were deparaffinized in xylenes and rehydrated through a graded alcohol series. Tissue sections were either stained with Harris hematoxylin solution and eosin Y solution (Sigma-Aldrich), or immunostained for FES expression. For immunofluorescent staining, antigen retrieval was performed in sodium citrate buffer using a microwave oven. Cells were then blocked in PBS containing 1% normal serum for 1 h and incubated overnight at 4 °C with an anti-Fes primary antibody (Santa Cruz Biotechnology, C-19; 1:250 dilution). Immunostained tissue was visualized with secondary antibodies conjugated to Alexa Fluor 594 (Invitrogen). Fluorescent images were recorded using a Nikon TE300 inverted microscope with epifluorescence capability and a SPOT CCD high-resolution digital camera and software (Diagnostic Instruments).

Bisulfite genomic DNA sequencing

Genomic DNA was extracted directly from paraffin-embedded thin sections of normal colonic epithelial tissue using the Pinpoint Slide DNA Isolation System (Zymo Research). Genomic DNA from 5-aza-2′-dC treated and untreated HT-29 cells was isolated using the PureLink Genomic DNA Purification Kit (Invitrogen). Genomic DNA aliquots were then treated with sodium bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research). The targeted region of the *FES* promoter [−104 to +126 relative to the first transcription initiation site (He et al., 1996)] was then amplified using a fully-nested Hot-Start Taq (Qiagen) PCR protocol. For the first PCR round, 2 μl of bisulfite-treated genomic DNA was amplified using the following PCR conditions: 95 °C for 15 min, followed by 35 cycles at 94 °C for 1 min, 50 °C for 30 sec, 72 °C for 1 min plus a final extension at 72 °C for 10 min. Primers designed to recognize the bisulfite-modified 362 bp (−183 to +180) region of the *FES* promoter were5′-GTTGGGTTATTTTTTTTCGGTT-3′ (sense) and 5′-TAAATAAATCTCTAACCCTC-3′ (antisense). For the nested PCR, the following PCR conditions were used: 95 °C for 15 min, followed by 35 cycles at 94 °C for 1 min, 56 °C for 30 sec, and 72 °C for 1 min with a final extension at 72 °C for 10 min. Nested primers designed to amplify within the first round PCR product were 5′-CCCAAGCTTGGAGTAGGGGGGTTGGTAGG-3′ (sense) and 5′-CCCGAATTCCCTACTCTACCCCTACCTACC-3′ (antisense), which contain *Hind*III and *Eco*RI restriction sites, respectively. The PCR products (257 bp: −104 to +126) were then subcloned into pcDNA3.1(+) (Invitrogen) and used to transform DH5α *E. coli*. Plasmid DNA was isolated from individual colonies and sequenced to determine the methylation status of the CpG dinucleotides within this proximal *FES* promoter region (He et al., 1996).

Plasmid construction and *in vitro* methylation

A previously characterized minimal *FES* promoter region (−425 to + 91) (He et al., 1996) was amplified using the following primers, which contain *Nhe*I and *Hind*III restriction enzyme sites, respectively: 5′-CCCGCTAGCAATTCCGTGAGGTGGGGAGGG-3′ (sense) and 5′-CCCAAGCTTGTACCCGCACGGGCAGCTGCT-3′ (antisense). The resulting PCR product was subcloned into pcDNA3.1(+), and the nucleotide sequence was verified. This *FES* minimal promoter was then digested from pcDNA3.1(+), purified, and aliquots incubated in the presence or absence of *Sss*I methylase (New England BioLabs) (Pogribny et al., 2000). The efficiency of the methylation reaction was verified by resistance to cleavage by the methylation-sensitive restriction enzyme *Bstu*I. The methylated and unmethylated *FES* promoter regions were then purified and ligated into the pGL4.14 Luciferase Assay Vector (Promega). The pGL4.14 vector contains the firefly luciferase reporter gene, but lacks promoter and enhancer sequences. The pGL4.14 parent vector lacking a promoter insert served as the negative control. This approach was based on previous work by Pogribny and colleagues (Pogribny et al., 2000).

Transient transfection and luciferase assay

The ligation reactions from the methylated and unmethylated *FES* promoter fragments and pGL4.14 were combined with 3 μl of Fugene 6 in 50 μl of Opti-MEM (Invitrogen). To normalize for transfection efficiency, the ligation products were cotransfected with 0.12 μg of pGL4.74 *Renilla* luciferase vector. Following a 30 min room temperature incubation, the Fugene 6/DNA transfection complex was added to 2×10^5 293T cells. Forty-eight h later, the cells were washed with PBS, harvested by scraping into a passive lysis buffer (Promega), and lysed according to the manufacturer's protocol. Cell extracts were assayed in a Victor2 1420 multilabel counter (Perkin Elmer) for firefly and *Renilla* luciferase activity using the Dual-Luciferase Reporter Assay System (Promega).

RESULTS

FES transcripts are reduced or absent in CRC cells

Recent work from our laboratory established that FES protein expression is reduced or absent in CRC cell lines as well as primary tumor samples (Delfino et al., 2006b). To determine whether loss of FES protein correlates with loss of *FES* mRNA, RT-PCR experiments were conducted on RNA isolated from seven CRC cell lines. RNA isolated from the human myeloid leukemia cell lines TF-1 and K-562 served as positive and negative controls, respectively (Heydemann et al., 1996; Smithgall et al., 1998). As shown in Figure 1A, *FES* expression was significantly reduced or absent in the CRC cell lines Caco-2, DLD-1, HT-29, SNU-1040, and SW-480, as well as the control K-562 cell line as measured by RT-PCR amplification of the 3′ end of the *FES* transcript. Surprisingly, 3′ *FES* RT-PCR products were observed in COLO 320 and HCT 116 at levels similar to TF-1 cell positive control, possibly suggesting that post-transcriptional events are responsible for the lack of Fes protein previously reported for these two CRC cell lines (Delfino et al., 2006b). However, amplification of a 5′ portion of the *FES* transcript (exon 1 through FCH domain in exon 2) established *FES* expression was significantly reduced or absent in all of the CRC cell lines (Figure 1B), including COLO 320 and HCT 116 (Figure 1B). These observations imply that the 3′ PCR products observed in Figure 1A with COLO 320 and HCT 116 cells are derived from incomplete transcripts and are non-functional. To verify the integrity of the *FES* gene, southern blot analysis was performed as previously described (Smithgall et al., 1991). Figure 1C shows that probes specific for the 5′ and 3′ ends of the *FES* gene detected restriction fragments of the expected lengths (5′, 5.05 kb; 3′, 4.16 kb) for all of the samples tested, confirming that the *FES* gene is present and not grossly rearranged in the CRC or myeloid cell lines used in this experiment.

The *FES* promoter harbors a potential CpG island

Normally unmethylated CpG islands may become hypermethylated in tumors, leadings to irreversible inhibition of gene expression (Jones and Takai, 2001; Christman, 2002). Previous findings have suggested that a CpG island may exist at the 5′ end of the human *FES* locus (Alcalay et al., 1990). To determine whether a CpG island exists within the *FES* promoter, the DNA sequence was analyzed using the EMBOSS CpGPlot program, which detects regions of genomic DNA sequences that are rich in the CpG dinucleotide pattern (Rice et al., 2000). As shown in Figure 2A, a 375 bp CpG island was identified in the human *FES* promoter at nucleotide positions −249 to +126 relative to the first transcription initiation site (He et al., 1996). Feline and mouse *FES* promoter sequences were also analyzed for the presence of CpG islands. Figure 2B shows that a putative 323 bp CpG island is also present within feline *FES* promoter, while sequence analysis failed to detect a CpG island within the mouse *FES* promoter (data not shown). Whether or not promoter methylation is a unique regulatory feature of the human and cat promoters and does not operate in mice will require further investigation. For the human *FES* promoter, note the high density of CpG dinucleotides (red) located near the transcription initiation sites (underlined) (Figure 2C). Methylation of even a small core region near a transcription start site is often sufficient for gene silencing (Jones and Takai, 2001).

Demethylation restores expression of functional *FES* transcripts

To establish a role for DNA methylation in the repression of *FES* gene expression observed in Figure 1, the same panel of CRC and myeloid leukemia cell lines was treated with the demethylation reagent 5-aza-2′-deoxycytidine (5-aza-2′-dC) followed by RT-PCR analysis of the 3′ and 5′ regions of the *FES* transcript as in Figure 1. 5-aza-2′-dC treatment leads to rapid loss of DNA cytosine-C5 methyltransferase activity, because the enzyme becomes irreversibly bound to 5-aza-2′-dC upon incorporation into DNA [reviewed in (Christman, 2002)]. As shown in Figure 3A, RT-PCR analysis revealed that the 3′ region of the *FES* transcript was restored in Caco-2, DLD-1, HT-29, SNU-1040, SW-480, and K-562 cells following four-day treatment with 5-aza-2′-dC. In addition, RT-PCR products corresponding to the 5′end of the *FES* transcript were restored in all seven CRC cell lines as well as K562 cells upon 5-aza-2′-dC treatment, suggesting that functional transcripts are now present in each of these cell lines. The nucleotide sequences of all *FES* RT-PCR products were confirmed (data not shown).

To determine whether the *FES* RT-PCR products were derived from functional mRNA transcripts, lysates from 5-aza-2′-dC-treated cells were examined for FES protein by immunoprecipitation followed immunoblotting. As shown in Figure 3C, 5-aza-2′-dC treatment restored FES protein in Caco-2, DLD-1, HT-29, SNU-1040, SW-480, COLO 320, HCT 116, and K-562 cells, demonstrating the functionality of the *FES* transcripts. Regarding the two cells lines that exhibited 3′ but not 5′ transcripts in Figure 1, no truncated FES protein products were observed in COLO 320 suggesting the 3′ transcripts were not functional. On the other hand, two FES truncation variants at ~90 and 92 kDa were observed in HCT 116 cells, suggesting that the observed 3′ RT-PCR products are amplified from partial *FES* transcripts. TF-1 cells were used as a positive control for *FES* protein expression in this experiment. These results demonstrate that expression of functional *FES* transcripts in colorectal cancer cell lines, as well as K-562 CML cells, is restored in response to treatment with a DNA methyltransferase inhibitor.

The *FES* promoter is extensively methylated in CRC cells

We next investigated whether the putative CpG dinucleotides predicted to lie within the *FES* promoter were hypermethylated in CRC cell lines. First, we established the baseline methylation pattern of the *FES* promoter under physiological conditions by performing

sodium bisulfite sequencing on genomic DNA isolated from normal human colonic epithelium. For these experiments, thin sections of formalin-fixed paraffin-embedded normal human colon tissue were immunostained to confirm FES expression. A representative section is shown in Figure 4A, and displays both normal colonic microanatomy as well as strong epithelial staining for FES protein. Genomic DNA was isolated directly from a serial section of this sample, subjected to bisulfite treatment, and the *FES* promoter sequence surrounding the transcription start site was amplified by PCR. The PCR product was then subcloned into a plasmid vector and 26 individual clones were sequenced. The methylation status of the eleven CpG dinucleotides immediately upstream of the first *FES* transcription start site are presented in Figure 4B. These *FES* promoter CpG dinucleotides are largely demethylated, consistent with the strong Fes staining observed in colonic epithelium. Note that 13 of the 26 clones were completely unmethylated, with an additional eight sequences exhibiting only a single methylated CpG dinucleotide at a distance of 76 nucloetides or greater from the transcriptional start site.

To determine whether CpG dinucleotides near the start site of *FES* transcription are hypermethylated in colorectal cancer cells, DNA sequence analysis was performed on individual clones of bisulfite-treated genomic DNA isolated from both untreated and 5-aza-2′-dC-treated HT-29 cells as described above for normal colonic epithelium. As shown in Figure 5A, the proximal *FES* promoter from untreated HT29 cells was heavily methylated in comparison to normal colonic epithelium (Figure 4B), with only 3 of 30 clones unmethylated (10%) and many of the remaining clones showing multiple sites of methylation. In contrast, treatment with 5-aza-2′-dC induced a dramatic decrease in methylation at nine of eleven CpG sites, with the extent of methylation at seven of the sites reduced by more than 50% compared to the untreated control (Figure 5). Note that complete demethylation of all eleven CpG sites was observed in 13 of 34 clones (38%) from HT-29 cells treated with 5-aza-2′-dC. This reduction in promoter methylation in response to 5-aza-2′-dC treatment correlates with the re-expression of the *FES* gene (Figure 3), strongly suggesting that methylation directly controls *FES* gene expression.

In vitro methylation blocks *FES* promoter activity

To determine whether methylation directly impacts *FES* promoter activity, an *in vitro* methylation assay was performed using the dual-luciferase reporter assay. A previously defined minimal *FES* promoter (−425 to +91) with robust activity (He et al., 1996) was methylated *in vitro* using the *Sss*I methylase, and ligated upstream of the firefly luciferase coding sequence in the pGL4.14 vector. The efficiency of the methylation reaction was verified by resistance to *Bstu*I restriction enzyme cleavage (data not shown). Human 293T epithelial cells were then transfected with the ligation products and cells extracts were assayed for firefly luciferase activity. Luciferase activity from the methylated *FES* promoter and vector control are expressed relative to activity of the unmethylated *FES* promoter. As shown in Figure 6, methylation completely blocked *FES* promoter activity, to the same level as the promoterless vector control. This result strongly implies that promoter methylation is one important mechanism that directly governs expression of the *FES* gene in colorectal cancer and possibly other tumor sites where *FES* is normally expressed (e.g., breast epithelium, myeloid cells) (Haigh et al., 1996; Smithgall et al., 1998; Greer, 2002; Delfino et al., 2006b).

DISCUSSION

Although *FES* has been historically viewed as a proto-oncogene because of its protein-tyrosine kinase activity, several recent reports have established a tumor suppressor function for *FES* in epithelial cancers (Sangrar et al., 2005; Delfino et al., 2006b). Greer and colleagues determined that null or kinase-inactivating *FES* mutations accelerated tumor

onset in a mouse breast epithelial cancer model system (Sangrar et al., 2005). Importantly, the kinetics of tumor onset in targeted *FES* null mice was restored with a *FES* transgene in this study, allowing direct attribution of the effect on tumor latency to *FES* gene loss. Recent work from our group has demonstrated that loss of Fes protein expression is a common feature of both CRC cell lines as well as primary colon tumor specimens (Delfino et al., 2006b). We also determined that re-expression of wild-type or activated Fes in the CRC cell lines HT-29 and HCT 116 suppressed transformed colony growth in soft agar (Delfino et al., 2006b). Furthermore, re-expression of wild-type or activated Fes in HCT 116 cells almost completely suppresses invasion through a matrigel matrix, without affecting cell proliferation or viability (J. Shaffer and T. Smithgall, unpublished observation). While these previous studies support a tumor suppressor role for FES in colorectal and other epithelial cancers, the mechanism responsible for the loss of FES expression in tumor cells has not been investigated.

Data presented here are the first to define a mechanism by which *FES* gene expression is repressed in colorectal cancer. First, we established that full-length *FES* transcripts are absent in seven independent colorectal cancer cell lines, suggesting that the loss of FES protein previously observed in these cell lines (Delfino et al., 2006b) results directly from down-regulation of *FES* gene expression (Figure 1). Based on EMBOSS CpGPlot identification of a CpG island in the human *FES* promoter (Figure 2), we hypothesized that methylation of CpG dinucleotides within the *FES* promoter downregulates FES expression in CRC cell lines. Using the potent demethylation agent 5-aza-2′-dC, we re-established *FES* gene expression in each CRC cell line (Figures 3A and 3B). These data directly implicate promoter methylation as a key mechanism governing *FES* transcription in colorectal cancer cell lines. Treatment with 5-aza-2′-dC also restored expression of full-length (93 kDa) *FES* protein in each CRC cell line and in K562 CML cells, demonstrating that the RT-PCR products were derived from functional *FES* transcripts (Figure 3C). Of interest is the observation that truncated variants of FES were observed in untreated HCT 116 cells. However, full-length FES was only expressed in HCT 116 cells upon 5′-aza′-2-dC treatment, suggesting that expression of the full-length protein is controlled by promoter methylation in this cell line.

In addition to the CRC cell lines, we also observed that 5-aza-2′-dC treatment restored functional *FES* transcripts in the cell line K-562, which was derived from the blast crisis phase of chronic myelogenous leukemia (Lozzio et al., 1981). Previous work has established that *FES* expression is undetectable in K-562 cells, despite being of myeloid origin (Smithgall et al., 1988; Yu et al., 1989; He et al., 1996) and having an intact *FES* locus (Smithgall et al., 1991). Re-introduction of *FES* has been shown to cause growth suppression and differentiation in K562 cells, suggesting a tumor suppressor function for *FES* in CML as well. Consistent with our observations, Alcalay et al. reported that the *FES* promoter was hypomethylated in the myeloid leukemia cell lines HL-60, KG-1, and U937 (Alcalay et al., 1990), all of which strongly express FES (Smithgall et al., 1988).

In order to attribute *FES* gene downregulation to methylation of specific CpG dinucleotides within the *FES* promoter CpG island, we performed sodium bisulfite sequencing on the *FES* promoter from 5-aza-2′-dC-treated HT-29 cells. Using the methylation pattern of CpG dinucleotides from the *FES* promoter in normal colonic epithelial cells for comparison (Figure 4B), we found that several CpG sites within the *FES* promoter were heavily methylated in HT-29 cells (Figure 5A). These sites consistently exhibited reduced methylation following 5-aza-2′-dC treatment (Figure 5B). The actual degree of demethylation is most likely an underestimate, as 5-aza-2′-dC inhibits DNA (cytosine-C5) methyltransferase activity but does not remove pre-existing methylated cytosine residues (Christman, 2002). These methylated CpG dinucleotides lie in regions that can inhibit *FES*

gene transcription through one of two mechanisms. First, transcription factor binding may be inhibited by methylated CpG dinucleotides. While transcription factors controlling *FES* gene expression in colonic epithelial cells are not known, factors that regulate *FES* in myeloid cells have been extensively characterized. These include the ubiquitous transcription factor Sp1, the hematopoietic cell-specific factor PU.1/Spi-1, and a *FES* expression factor (FEF) that is not present in human epithelial cells (Ray-Gallet et al., 1995; Heydemann et al., 1996; Heydemann et al., 1997). Note that the DNA binding and transcriptional activities of Sp1, whose consensus binding site contains a central CpG site, are not influenced by methylation (Holler et al., 1988; Harrington et al., 1988). However, methylation may influence the DNA binding and transcriptional activities of tissue-specific transcription factors that drive *FES* expression both in myeloid and epithelial cells. A second possible mechanism by which promoter methylation down-regulates *FES* expression may involve methylation-dependent recruitment of nucleoprotein factors such as the methyl-CpG-binding proteins MeCP1 and MeCP2, which in turn deny access to transcription factors (Meehan et al., 1989; Boyes and Bird, 1991; Lewis et al., 1992; Cross et al., 1997; Nan et al., 1997). Future studies will define the precise mechanism by which methylation inhibits *FES* expression.

Data presented here also provide direct evidence that methylation governs *FES* promoter activity. *In vitro* methylation of a robustly active *FES* promoter (He et al., 1996) that mimics methylation patterns observed by bisulfite sequencing of HT-29 cell genomic DNA completely blocked the activity of the *FES* promoter in a reporter gene assay (Figure 6). This result suggests that methylation directly governs *FES* gene expression in CRC cell lines.

DNA methylation is a well-documented epigenetic mechanism altering gene expression in a variety of tumor types. Hypermethylation of tumor suppressor genes effectively abolishes their transcription, while hypomethylation of proto-oncogenes increases their transcription (Jones and Baylin, 2002; Herman and Baylin, 2003). In colorectal cancers, hypermethylation is a frequent event resulting in the silencing of well known tumor suppressors such as *P16*/*CDKN2A*/*INK4A* and *P14*/*ARF* (Herman et al., 1995; Robertson and Jones, 1998; Issa, 2000; Kondo and Issa, 2004; Mori et al., 2006). Here, we identify for the first time a candidate protein-tyrosine kinase tumor-suppressor gene that is hypermethylated in colorectal cancers. Through demethylation treatment, sodium bisulfite sequencing, and *in vitro* methylation assays, we have established that loss of *FES* expression in colorectal cancers may be due in part to methylation of CpG sites within the *FES* promoter. Selective re-expression of the *FES* tyrosine kinase gene with demethylation agents or other small molecules may be of value in CRC therapy.

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References

Alcalay M, Antolini F, Van de Ven WJM, Lanfrancone L, Grignani F, Pelicci PG. Characterization of human and mouse c-*fes* cDNA clones and identification of the 5′ end of the gene. Oncogene. 1990; 5:267–275. [PubMed: 2179816]

Bardelli A, Parsons DW, Silliman N, Ptak J, Szabo S, Saha S, Markowitz S, Willson JK, Parmigiani G, Kinzler KW, Vogelstein B, Velculescu VE. Mutational analysis of the tyrosine kinome in colorectal cancers. Science. 2003; 300:949. [PubMed: 12738854]

Baylin SB, Ohm JE. Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? Nat Rev Cancer. 2006; 6:107–116. [PubMed: 16491070]

Boyes J, Bird A. DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. Cell. 1991; 64:1123–1134. [PubMed: 2004419]

Cheng HY, Rogers JA, Dunham NA, Smithgall TE. Regulation of c-Fes tyrosine kinase and biological activities by N-terminal coiled-coil oligomerization domains. Mol Cell Biol. 1999; 19:8335–8343. [PubMed: 10567558]

Cheng HY, Schiavone AP, Smithgall TE. A point mutation in the N-terminal coiled-coil domain releases c-Fes tyrosine kinase activity and survival signaling in myeloid leukemia cells. Mol Cell Biol. 2001; 21:6170–6180. [PubMed: 11509660]

Christman JK. 5-Azacytidine and 5-aza-2 ′-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. Oncogene. 2002; 21:5483–5495. [PubMed: 12154409]

Cross SH, Meehan RR, Nan X, Bird A. A component of the transcriptional repressor MeCP1 shares a motif with DNA methyltransferase and HRX proteins. Nat Genet. 1997; 16:256–259. [PubMed: 9207790]

Delfino FJ, Shaffer JM, Smithgall TE. The KRAB-associated co-repressor KAP-1 is a coiled-coil binding partner, substrate, and activator of the c-Fes protein-tyrosine kinase. Biochem J. 2006a; 399:141–150. [PubMed: 16792528]

Delfino FJ, Stevenson HM, Smithgall TE. A growth-suppressive function for the c-Fes protein-tyrosine kinase in colorectal cancer. J Biol Chem. 2006b; 281:8829–8835. [PubMed: 16455651]

Fang JY, Lu J, Chen YX, Yang L. Effects of DNA methylation on expression of tumor suppressor genes and proto-oncogene in human colon cancer cell lines. World J Gastroenterol. 2003; 9:1976–1980. [PubMed: 12970888]

Feldman RA, Lowy DR, Vass WC, Velu TJ. A highly efficient retroviral vector allows detection of the transforming activity of the human c-*fps*/*fes* proto-oncogene. J Virol. 1989; 63:5469–5474. [PubMed: 2685357]

Greer P, Haigh J, Mbamalu G, Khoo W, Bernstein A, Pawson T. The Fps/Fes protein-tyrosine kinase promotes angiogenesis in transgenic mice. Mol Cell Biol. 1994; 14:6755–6763. [PubMed: 7523858]

Greer PA. Closing in on the biological functions of Fps/Fes and Fer. Nature Rev Mol Cell Biol. 2002; 3:278–289. [PubMed: 11994747]

Greer PA, Meckling-Hansen K, Pawson T. The human c-*fps*/*fes* gene product expressed ectopically in rat fibroblasts is nontransforming and has restrained protein-tyrosine kinase activity. Mol Cell Biol. 1988; 8:578–587. [PubMed: 3352601]

Haigh J, McVeigh J, Greer P. The Fps/Fes tyrosine kinase is expressed in myeloid, vascular endothelial, epithelial and neuronal cells and is localized to the trans-golgi network. Cell Growth and Differentiation. 1996; 7:931–944. [PubMed: 8809411]

Harrington MA, Jones PA, Imagawa M, Karin M. Cytosine methylation does not affect binding of transcription factor Sp1. Proc Natl Acad Sci U S A. 1988; 85:2066–2070. [PubMed: 3281160]

He Y, Borellini F, Koch WH, Huang K-X, Glazer RI. Transcriptional regulation of c-*fes* in myeloid leukemia cells. Biochim Biophys Acta. 1996; 1306:179–186. [PubMed: 8634335]

Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med. 2003; 349:2042–2054. [PubMed: 14627790]

Herman JG, Merlo A, Mao L, Lapidus RG, Issa JP, Davidson NE, Sidransky D, Baylin SB. Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res. 1995; 55:4525–4530. [PubMed: 7553621]

Heydemann A, Boehmler JH, Simon MC. Expression of two myeloid cell-specific genes requires the novel transcription factor, c-*fes* expression factor. J Biol Chem. 1997; 272:29527–29537. [PubMed: 9368014]

Heydemann A, Juang G, Hennessy K, Parmacek MS, Simon MC. The myeloid-cell-specific *c-fes* promoter is regulated by Sp1, PU.1, and a novel transcription factor. Mol Cell Biol. 1996; 16:1676–1686. [PubMed: 8657143]

Holler M, Westin G, Jiricny J, Schaffner W. Sp1 transcription factor binds DNA and activates transcription even when the binding site is CpG methylated. Genes Dev. 1988; 2:1127–1135. [PubMed: 3056778]

Issa JP. The epigenetics of colorectal cancer. Ann N Y Acad Sci. 2000; 910:140–153. [PubMed: 10911911]

Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet. 2002; 3:415–428. [PubMed: 12042769]

Jones PA, Takai D. The role of DNA methylation in mammalian epigenetics. Science. 2001; 293:1068–1070. [PubMed: 11498573]

Kondo Y, Issa JP. Epigenetic changes in colorectal cancer. Cancer Metastasis Rev. 2004; 23:29–39. [PubMed: 15000147]

Lewis JD, Meehan RR, Henzel WJ, Maurer-Fogy I, Jeppesen P, Klein F, Bird A. Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. Cell. 1992; 69:905–914. [PubMed: 1606614]

Lionberger JM, Smithgall TE. The c-Fes protein-tyrosine kinase suppresses cytokine-independent outgrowth of myeloid leukemia cells induced by Bcr-Abl. Cancer Res. 2000; 60:1097–1103. [PubMed: 10706130]

Lozzio BB, Lozzio CB, Bamberger EG, Feliu AS. A multipotential leukemia cell line (K-562) of human origin. Proc Soc Exp Biol Med. 1981; 166:546–550. [PubMed: 7194480]

Meehan RR, Lewis JD, McKay S, Kleiner EL, Bird AP. Identification of a mammalian protein that binds specifically to DNA containing methylated CpGs. Cell. 1989; 58:499–507. [PubMed: 2758464]

Mori Y, Cai K, Cheng Y, Wang S, Paun B, Hamilton JP, Jin Z, Sato F, Berki AT, Kan T, Ito T, Mantzur C, Abraham JM, Meltzer SJ. A genome-wide search identifies epigenetic silencing of somatostatin, tachykinin-1, and 5 other genes in colon cancer. Gastroenterology. 2006; 131:797–808. [PubMed: 16952549]

Nan X, Campoy FJ, Bird A. MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. Cell. 1997; 88:471–481. [PubMed: 9038338]

Pogribny IP, Pogribna M, Christman JK, James SJ. Single-site methylation within the p53 promoter region reduces gene expression in a reporter gene construct: possible in vivo relevance during tumorigenesis. Cancer Res. 2000; 60:588–594. [PubMed: 10676641]

Ray-Gallet D, Mao C, Tavitian A, Moreau-Gachelin F. DNA binding specificities of Spi-1/PU.1 and Spi-B transcription factors and identification of a Spi-1/Spi-B binding site in the c-fes/c-fps promoter. Oncogene. 1995; 11:303–313. [PubMed: 7624145]

Rice P, Longden I, Bleasby A. EMBOSS: the European Molecular Biology Open Software Suite. Trends Genet. 2000; 16:276–277. [PubMed: 10827456]

Robertson KD, Jones PA. The human ARF cell cycle regulatory gene promoter is a CpG island which can be silenced by DNA methylation and down-regulated by wild-type p53. Mol Cell Biol. 1998; 18:6457–6473. [PubMed: 9774662]

Rogers JA, Cheng HY, Smithgall TE. Src homology 2 domain substitution modulates the kinase and transforming activities of the Fes protein-tyrosine kinase. Cell Growth Differ. 2000; 11:581–592. [PubMed: 11095247]

Sangrar W, Zirgnibl RA, Gao Y, Muller WJ, Jia Z, Greer PA. An identity crisis for fps/fes: oncogene or tumor suppressor? Cancer Res. 2005; 65:3518–3522. [PubMed: 15867340]

Smithgall TE, Johnston JB, Bustin M, Glazer RI. Elevated expression of the c-*fes* proto-oncogene in adult human myeloid leukemia cells in the absence of gene amplification. J Natl Cancer Inst. 1991; 83:42–46. [PubMed: 1984516]

Smithgall TE, Rogers JA, Peters KL, Li J, Briggs SD, Lionberger JM, Cheng H, Shibata A, Scholtz B, Schreiner S, Dunham NA. The c-Fes Family of Protein-Tyrosine Kinases. Critical Rev Oncogenesis. 1998; 9:43–62. [PubMed: 9754447]

Smithgall TE, Yu G, Glazer RI. Identification of the differentiation-associated p93 tyrosine protein kinase of HL-60 leukemia cells as the product of the human c-*fes* locus and its expression in myelomonocytic cells. J Biol Chem. 1988; 263:15050–15055. [PubMed: 3170574]

Ushijima T. Detection and interpretation of altered methylation patterns in cancer cells. Nat Rev Cancer. 2005; 5:223–231. [PubMed: 15719030]

Yu G, Smithgall TE, Glazer RI. K562 leukemia cells transfected with the human c-*fes* gene acquire the ability to undergo myeloid differentiation. J Biol Chem. 1989; 264:10276–10281. [PubMed: 2656706]

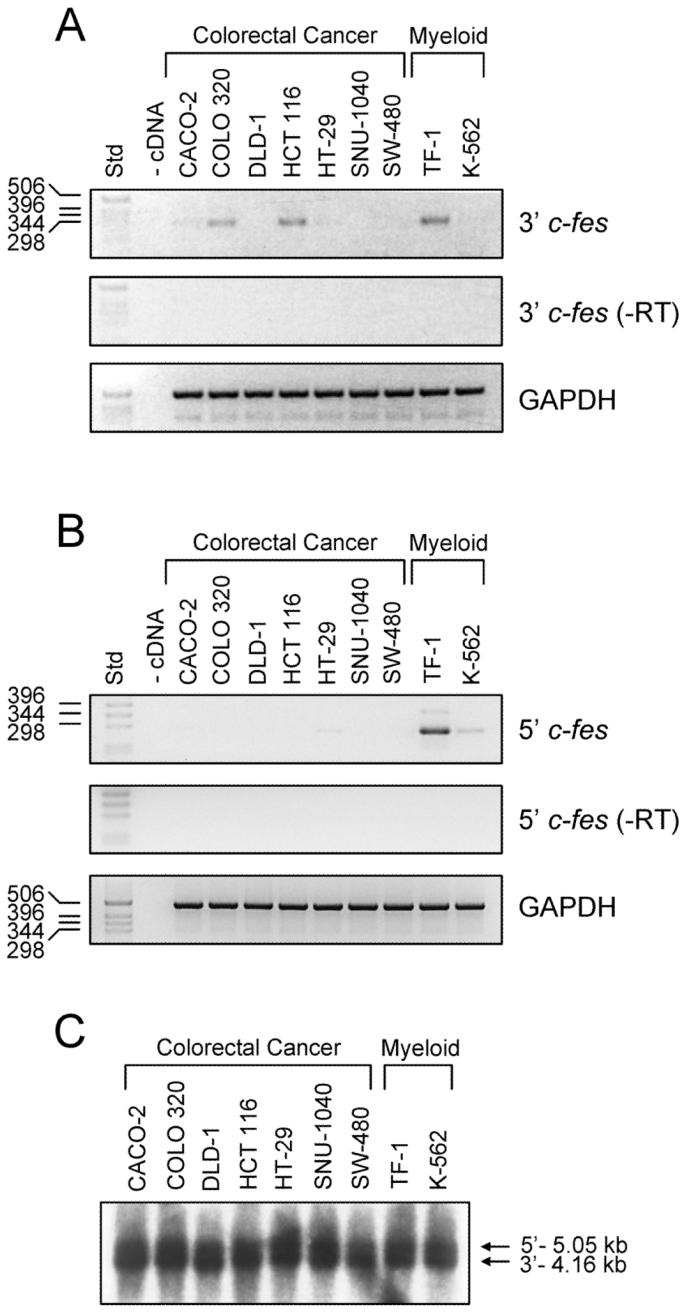

Figure 1.

Transcription of *FES* is low or absent in colorectal cancer cell lines. Total RNA and genomic DNA were isolated from colorectal cancer (Caco-2, COLO 320, DLD-1, HCT 116, HT-29, SNU-1040, and SW-480) and myeloid leukemia (TF-1 and K-562) cell lines for RT-PCR and southern blot analyses, respectively. A) RT-PCR analysis of *FES* (3′ region: 280 bp) and GAPDH (470 bp) mRNA levels. Control reactions lacking reverse transcriptase (-RT) are also shown. PCR reactions were analyzed after 35 cycles. Representative images of ethidium bromide-stained agarose gels are shown; images inverted for clarity. The experiment was repeated three times with comparable results. B) RT-PCR analysis of *FES* (5′ region: 266 bp) and GAPDH (470 bp) mRNA levels. Control reactions lacking reverse

transcriptase (-RT) are also shown. PCR reactions were analyzed after 40 cycles for *FES* and 35 cycles for GAPDH. Representative images of ethidium bromide-stained agarose gels (inverted for clarity) are shown. The experiment was repeated two times with comparable results. C) Southern blot analysis of the *FES* gene in colorectal cancer and myeloid leukemia cell lines. Genomic DNA (10 μg) from each cell line were digested with *Eco*RI and *Bam*HI, resolved on an agarose gel, and transferred to a nylon membrane. The membrane was probed with DNA fragments specific for the 5′ and 3′ ends of the *FES* gene (Smithgall et al., 1991). Comparable results were obtained from three independent experiments.

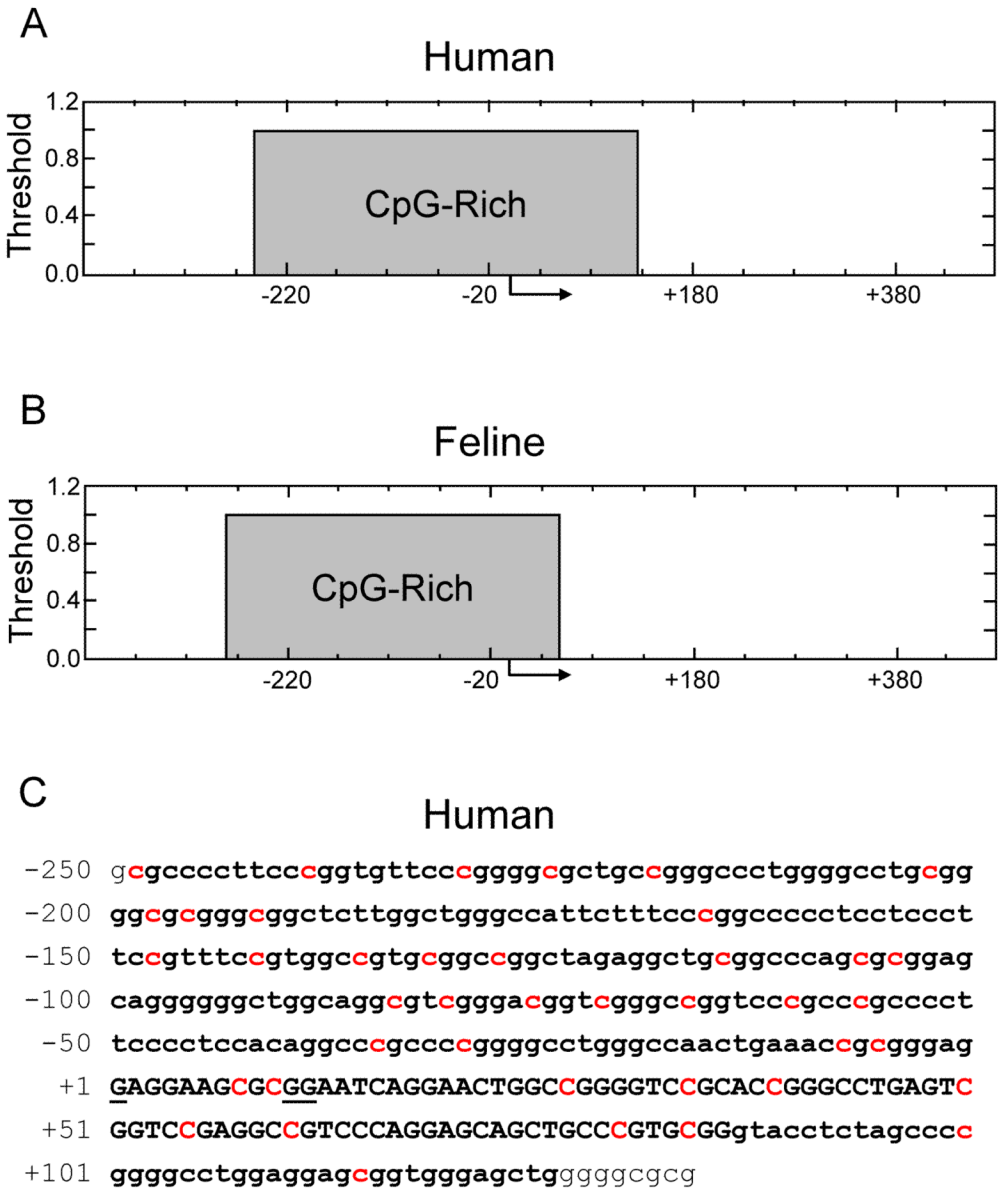

Figure 2.

Computational analysis reveals potential CpG islands within the human and feline *FES* promoters. A) A 375 bp CpG-rich region (−249 to +126 relative to the first transcription initiation site) was identified in the 1 kb human *FES* promoter region using the EMBOSS CpGPlot program (Rice et al., 2000). The position of the first transcriptional start site is indicated by the arrow. B) A potential 323 bp CpG island also exists with the feline *FES* promoter. C) Potentially methylated cytosines (red) are indicated within the human CpG island (bold). Exon 1 is indicated by upper case letters. The transcription initiation sites in exon 1 are underlined (He et al., 1996).

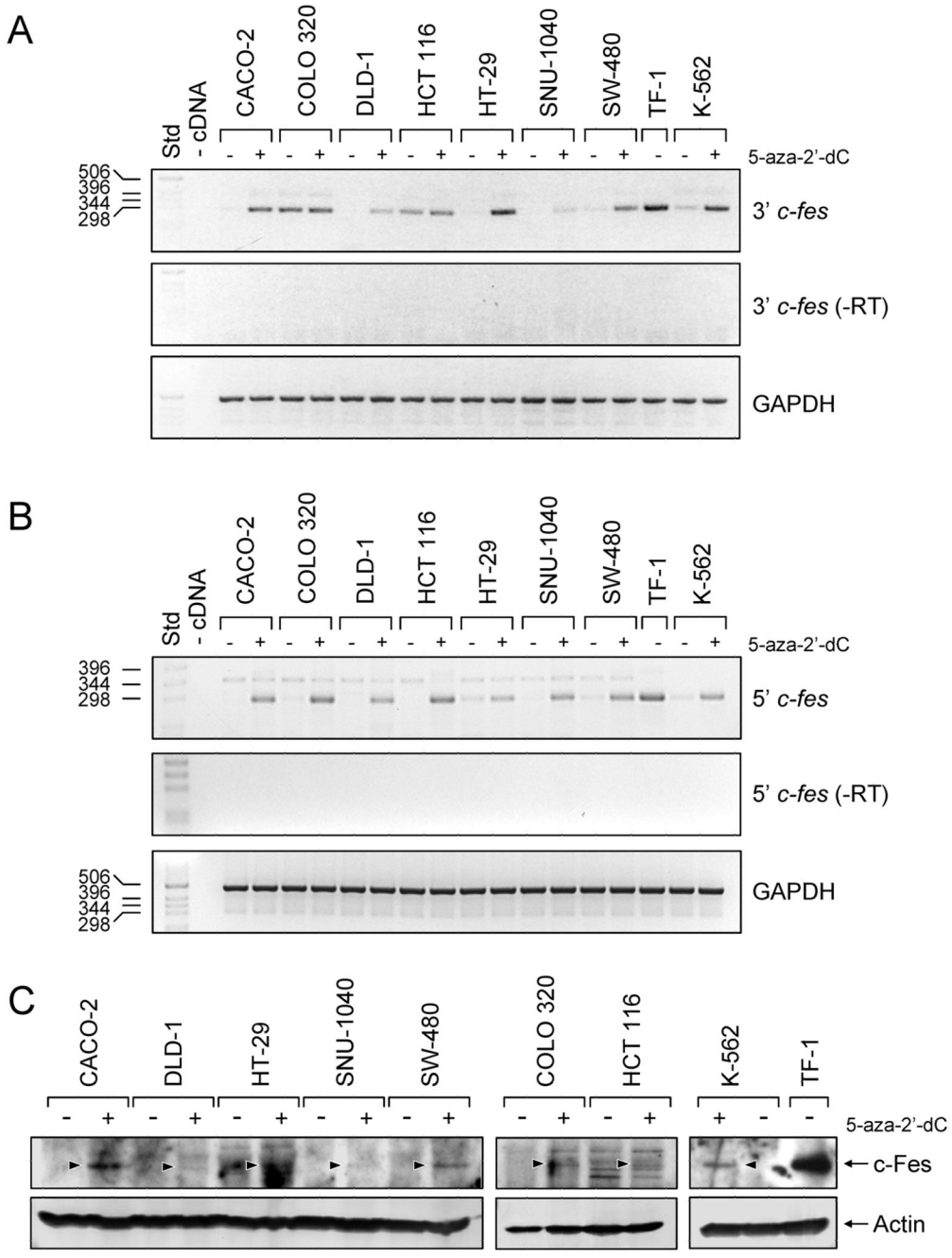

Figure 3.

5-aza-2′-deoxycytidine treatment restores functional *FES* transcripts in colorectal cancer cell lines. Colorectal cancer (Caco-2, COLO 320, DLD-1, HCT 116, HT-29, SNU-1040, and SW-480) and myeloid leukemia (TF-1 and K-562) cell lines were incubated with (+) or without (−) 10 μM 5-aza-2′-dC for four days. A) RT-PCR analysis of *FES* (3′ region: 280 bp) and GAPDH (470 bp) transcript levels from treated vs. untreated cells. Controls reactions lacking reverse transcriptase (-RT) are also shown. PCR reactions were analyzed after 35 cycles. Representative images of ethidium bromide-stained agarose gels are inverted for clarity. B) RT-PCR analysis of *FES* (5′ region: 266 bp) and GAPDH (470 bp) transcript levels from treated vs. untreated cells. Controls reactions lacking reverse transcriptase (-RT)

are also shown. PCR reactions were analyzed after 40 cycles for *FES* or 35 cycles for GAPDH. Representative images of ethidium bromide-stained agarose gels are inverted for clarity. C) FES kinase protein was immunoprecipitated from control and 5-aza-2′-dC treated cells using an antibody generated against its amino-terminal and SH2 regions. Immunoprecipitates were then immunoblotted with an antibody raised against the carboxyl-terminus of FES (upper panel). Fes was immunoprecipitated from TF-1 myeloid leukemia cells as a positive control (far right lane). The position of the immunoreactive 93 kDa FES bands from the 5-aza-2′-dC-treated cultures are indicated with the arrowheads. Cell lysates were blotted with an anti-actin antibody to ensure equivalent levels of input protein for each immunoprecipitation reaction (lower panel). All experiments were repeated two or three times with comparable results.

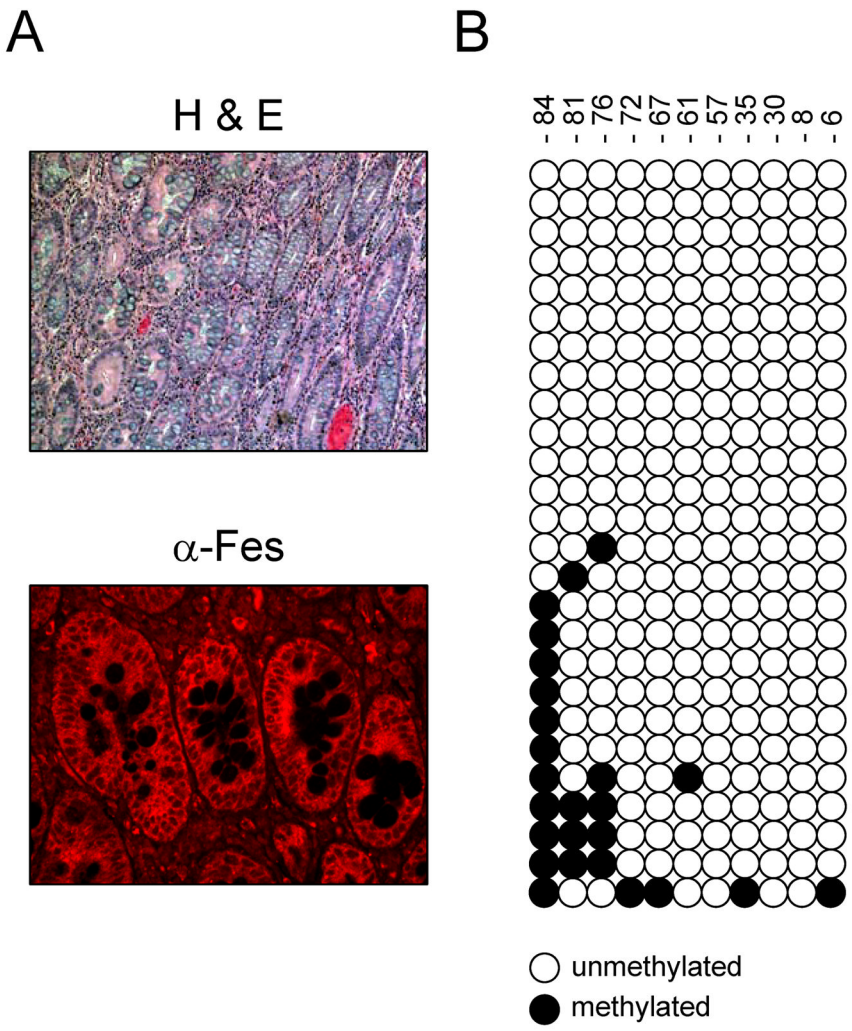

Figure 4.

FES protein expression in normal colonic epithelium correlates with hypomethylation of the *FES* promoter. A) Thin sections of formalin-fixed paraffin-embedded normal colonic epithelial tissue were stained with hematoxylin and eosin (H&E; 100X; top) or immunostained with FES antibodies and visualized with secondary antibodies conjugated to Alexa Fluor 594 (α-Fes; 400X; bottom). No immunofluorescence was observed without the primary Fes antibody (data not shown). B) Methylation status of the eleven CpG dinucleotides immediately preceding the first *FES* transcription initiation site. Genomic DNA was isolated directly from the normal colonic epithelial tissue sections and treated with sodium bisulfite. The *FES* promoter region was PCR-amplified and subcloned into the plasmid vector pcDNA3.1. The rows of circles summarize the bisulfite sequencing results from each of 26 independent clones. Open circles represent unmethylated CpG dinucleotides, while filled circles represent methylated CpG sites. The position of each CpG nucleotide relative to the first *FES* transcription initiation site is indicated at the top.

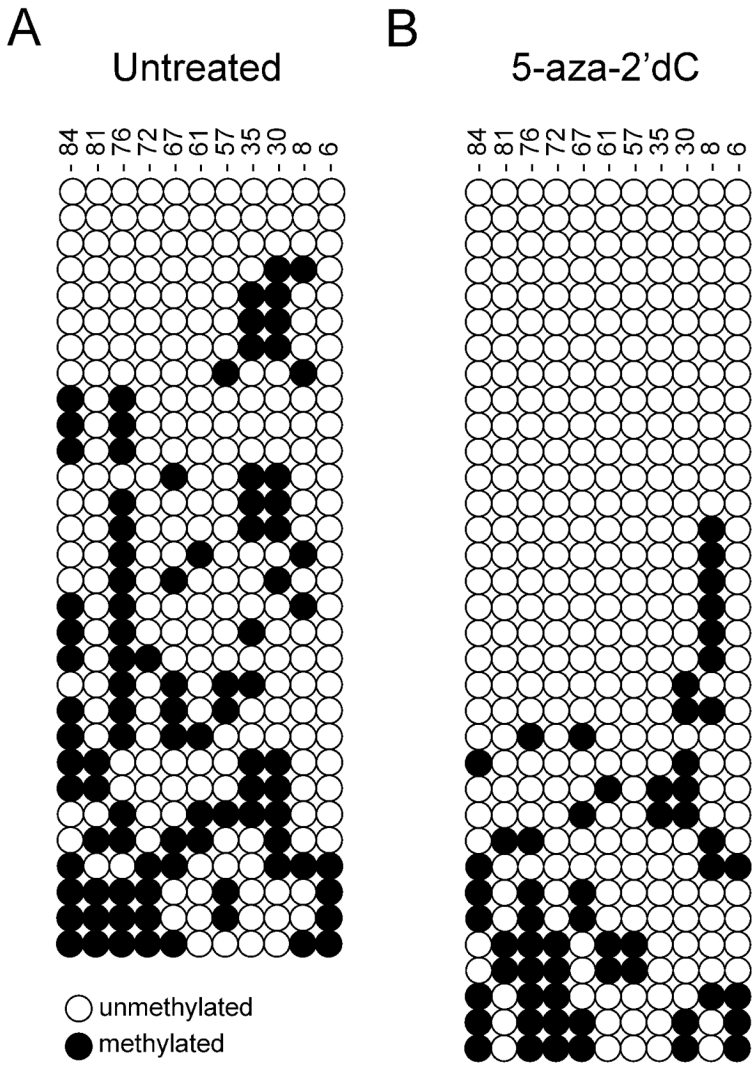

Figure 5.

Bisulfite sequencing reveals extensive *FES* promoter methylation in HT-29 cells which is reversed by 5-aza-2′-dC treatment. HT-29 cells were grown in the presence or absence of 15 μM 5-aza-2′-dC for eight days. Genomic DNA was isolated, treated with sodium bisulfite, and the *FES* promoter region was PCR-amplified and subcloned into the plasmid vector pcDNA3.1. A) Methylation status of the eleven CpG dinucleotides immediately preceding the first *FES* transcription initiation site in untreated HT-29 cells. The rows of circles summarize the bisulfite sequencing results from each of 30 independent clones. Open circles represent unmethylated CpG nucleotides, while filled circles represent methylated CpG sites. The position of each CpG nucleotide relative to the first *FES* transcription initiation site is indicated at the top. B) Methylation status of the proximal *FES* promoter in 5-aza-2′-dC treated HT-29 cells. Bisulfite sequence analysis of the *FES* promoter was performed as in part A, except 34 individual clones were sequenced. This entire experiment was repeated twice with comparable results.

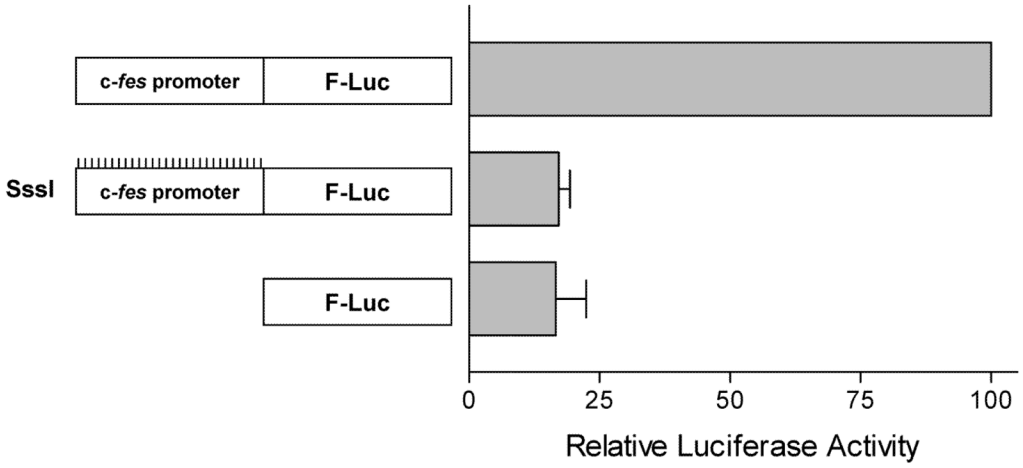

Figure 6.

Methylation regulates *FES* promoter activity *in vitro*. Relative firefly luciferase activity after *in vitro* methylation of *FES* promoter fragments using the Dual Luciferase Reporter Assay System (Promega). A minimal *FES* promoter region (−425 to + 91) was methylated *in vitro* using *Sss*I methylase. Control and methylated *FES* promoter regions were then purified, ligated into the pGL4.14 Luciferase Assay Vector, and transfected into 293T cells along with the *Renilla* luciferase control vector. Forty-eight h later, the cells were washed and assayed for luciferase activity. Firefly luciferase activity was normalized to the *Renilla* luciferase control, and activity from the methylated *FES* promoter and vector control are expressed relative to the unmethylated *FES* promoter (set to 100%). Tick marks represent methylation of *FES* promoter CpG dinucleotides by the *Sss*I methylase. Data presented represent the mean ± S.D. of three independent experiments.