Methylation of a Novel CpG Island of Intron I Is Associated With Steroidogenic Factor I Expression in Endometriotic Stromal Cells

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Abstract

Steroidogenic factor I (SF-I), a transcriptional factor essential for estrogen biosynthesis, is undetectable in endometrial stromal cells and aberrantly expressed in endometriotic stromal cells. **Objective:** We tried to gain further insight into the mechanism for differential SF-I expression in endometrial and endometriotic stromal cells. **Design:** We had previously identified a novel CpG island in SF-I, which is located in the downstream intron I region. Here, we evaluated the methylation status of this CpG island. **Patients:** We obtained the eutopic endometrium from disease-free participants ($n = 8$) and the walls of cystic endometriosis lesions of the ovaries from another group of participants ($n = 8$). None of the patients had received any preoperative hormonal therapy. **Interventions:** Stromal cells were isolated from these 2 types of tissues and subjected to DNA bisulfite treatment and sequence analysis. **Results:** The SF-I messenger RNA (mRNA) levels in endometriotic stromal cells were significantly higher than those in endometrial stromal cells. Bisulfite sequencing showed strikingly increased methylation of a 1-kbp region around the previously identified CpG island in endometriotic cells compared with endometrial cells ($P < .001$). A strong correlation between SF-I mRNA levels and percentage methylation of the intron I region of the *SF-I* gene was observed in endometriotic cells (Spearman correlation coefficient, .96; $P < .001$). **Conclusions:** Methylation of the intron I region of the SF-I gene is associated with its expression in endometriotic cells. This CpG island therefore plays an important role in regulating SF-I expression.

Keywords

SF-I, endometriosis, DNA methylation, intron, CpG island

Introduction

Endometriosis is a gynecological disease characterized by the presence of endometrial glandular and stromal cells in the extra-uterine environment.¹ These extrauterine glands and stroma, called endometriotic lesions, can be found on the ovaries and on the surfaces of pelvic cavity organs.² Endometriosis, although not malignant, occurs spontaneously in women and nonhuman primates that menstruate, causing pain and infertility. The high prevalence and severe outcomes associated with this disease have made endometriosis a major public health concern in modern society. Although the mechanism responsible for the initiation of this disease remains unclear, aberrant production of estrogen by ectopic endometriotic implants has been demonstrated to be a critical factor associated with endometriosis.^{3,4}

The biosynthesis of estrogen is controlled by 2 rate-limiting proteins, namely steroidogenic acute regulatory protein (StAR) and aromatase. Bulun and others demonstrated that abundant expression of steroidogenic genes including StAR gave rise to local estrogen production in endometriotic tissue.^{3,5,6} Aromatase and StAR are regulated by a nuclear receptor termed steroidogenic factor 1 (SF-1) in endometriotic stromal cells, which is a

member of the nuclear receptor superfamily.⁵ The SF-1 is also known as adrenal 4-binding protein (Ad4BP) and is encoded by the *NR5A1* gene in humans.^{7,8} The SF-1 is essential for normal development and function of steroid-producing gonads and adrenal glands.^{9,10} The SF-1 is expressed in endometriotic tissue but not in its normal counterpart, the eutopic endometrium.¹¹

We previously demonstrated aberrant methylation of the promoter and exon 1 region of the *NR5A1* gene in SF-1-negative cells (normal endometrial cells): complete repression of SF-1 transcriptional activity was observed in these cells.¹¹ Next, we found that methylation of the CpG island that spans

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Figure 1. Schematic diagram showing the CpG island in the intron 1 region of steroidogenic factor 1. The transcription start site is indicated as +1. Upper black bar, predicted CpG island; lower black bars, bisulfite sequencing fragment identified from assay 1, assay 2, and assay 3.

from exon 2 to intron 3 in the coding region of the *SF-1* gene led to aberrant SF-1 expression in endometriotic cells.¹²

We wished to gain further insight into the mechanism underlying the differential expression of this gene in endometrial and endometriotic cells. Therefore, we focused on intron 1 of the *SF-1* gene, because a novel and large CpG island has been mapped within this region, with length spanning approximately 1000 bp. To evaluate the function of this island in the regulation of the *SF-1* gene, we examined methylation features of this CpG island and investigated the correlation between SF-1 expression and methylation status in endometriotic stromal cells and endometrial stromal cells.

Materials and Methods

Isolation and Culture of Endometrial and Endometriotic Stromal Cells

Eutopic endometrium and ectopic endometrium from the cyst walls of ovarian endometriomas were obtained immediately after surgery from women (eutopic endometrium, $n = 8$; ectopic endometrium, $n = 8$) who underwent hysterectomy for benign indications other than endometriosis. None of the patients had received preoperative hormonal therapy, and all samples were histologically confirmed. The phase of menstrual cycle was determined by both preoperative history and histologic evaluation of the endometrium. Half of the tissue samples were in the proliferative phase and the other half in the secretory phase in both groups. Eutopic endometrial samples were obtained from women undergoing hysterectomy for cervical dysplasia or uterine leiomyoma. The average age of the patients was 40.75 ± 3.37 years (endometrium group) and 38.88 ± 2.95 years (endometriosis group), and there were no significant differences between the 2 groups with respect to age or menstrual phase. Written informed consent for obtaining the tissue was obtained from the patients prior to surgery.

Stromal cells were isolated using a previously described protocol, with minor modifications.¹³ Briefly, tissues were minced, digested with collagenase (Sigma, St Louis, Missouri) and DNase (Sigma) at 37°C for 30 minutes and then with collagenase, DNase, pronase (Sigma), and hyaluronidase (Sigma) for an additional 20 minutes. Epithelial and stromal cells were separated by filtration through 70- and 20-mm sieves. These

stromal cells were resuspended in Dulbecco modified Eagle medium/F12 1:1 (GIBCO/BRL, Grand Island, New York) containing 10% fetal bovine serum and grown in a humidified atmosphere containing 5% CO₂ at 37°C. Both endometriotic and endometrial stromal cells passaged once after primary culture.

RNA Extraction and Quantitative Analysis by Real-Time Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated from stromal cells using TRIzol (Sigma) according to the manufacturer's protocol. One microgram of total RNA was used to generate complementary DNA with the Superscript III first-strand synthesis system (Invitrogen, Carlsbad, California). Real-time quantitative polymerase chain reaction was performed using the ABI 7900 Sequence Detection system and the ABI Taqman Gene Expression system (Applied Biosystems, Foster City, California) to quantify SF-1 and human 18S RNA. The 18S values were used for normalization. The following primers were used for the SF-1 coding region: forward, 5'-CTGGAGCCGGATGAGGAC-3'; reverse, 5'-ACCTGGCGG TAGATGTGGT-3'. The 18S primers were forward, 5'-AGGAA TTCCAGTAAGTG-CG-3'; reverse, 5'-GCCTCACTAAAC-CATCCAA-3'.

DNA Bisulfite Treatment and Sequencing Analysis

DNA was isolated from cells using a DNeasy Kit (Qiagen, Valencia, California) according to the product instructions. The isolated DNA was treated with bisulfite using the EZ DNA Methylation Gold kit (Zymo Research, Orange, California) and then subjected to PCR amplification. Primers for the modified DNA were generated. The following primers for human SF-1 were used for assay 1: forward, 5'-GGGT GGGGATTGGATTAGAGATT-3'; reverse, 5'-CTTCCRC CTCCAAATTTTCTCTCC-3'. The primers for assay 2 were as follows: forward, 5'-GATTTTGAGAAAAAGAGATGGG TT-3'; reverse, 5'-CAATATCTATATCTCTAAC-ACTCTC-3', and the primers for assay 3 were as follows: forward, 5'-GTGTAG TGTTTGTGTTTGTAT-3'; reverse, 5'-AACCCATCTCTT TTTCTCAAAATC-3'.

The PCR thermal cycling conditions were as follows: 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 2 minutes, and elongation

Figure 2. A, DNA methylation status of the intron 1 region of steroidogenic factor 1 (SF-1; assay 2) in endometrial and endometriotic stromal cells. Open and filled circles represent unmethylated and methylated cytosines, respectively. The numbers on the top indicate the positions of cytosine residues of CpG dinucleotides relative to the transcription start site (+1), and the numbers, 1 to 8 on the left, represent primary cultured stromal cells from different participants in the 2 groups. B, Percentage methylation of intron 1 region of the SF-1 in endometrial and endometriotic cells. There was a significant difference in methylation at this site between the 2 groups of cells (* $P < .001$; Student t test).

at 72°C for 2 minutes, followed by a final extension at 72°C for 7 minutes. The PCR products (assay 1: 185-bp region spanning 14 CpG dinucleotides [CpGs]; assay 2: 225-bp region spanning 18 CpGs; and assay 3: 577-bp region spanning 70 CpGs) were cloned into the pGEM-Teasy vector (Promega, Madison, Wisconsin). Following transformation, 6 to 8 clones with the right insert were randomly picked from each PCR reaction and sequenced on an Applied Biosystems 377 instrument.

Statistical Analysis

Percentage methylation of each clone obtained from each of the 8 patients in each group was used for statistical analysis. The data were analyzed using Student t test with statistical

significance set at $P < .05$. Spearman's coefficient was calculated for the correlation between SF-1 messenger RNA (mRNA) levels and percentage methylation.

Results

Methylation of the CpG Island in the Intron 1 Region of SF-1

We focused on a 1-kb region around the CpG island flanking the intron 1 region of the *SF-1* gene in <http://www.urogen-e.org/methprimer/index1.html>. The criteria for a CpG island include a length of more than 500 bp, GC content higher than 50%, and observed CpG to expected CpG ratio higher than

Figure 3. Correlation between percentage methylation of intron 1 region of the steroidogenic factor 1 (SF-1; assay 2) and SF-1 mRNA expression (logarithmic scale) in the 8 endometriotic stromal cell samples. A significant correlation was observed between the 2 groups, with a Spearman correlation coefficient value of .96 ($P < .001$).

0.60¹⁴ (Figure 1). We amplified 3 different parts of this CpG island after bisulfite modification. Differences in methylation status between endometrial and endometriotic stromal cells were only found in the middle part of this CpG island (assay 2: 225-bp region spanning 18 CpGs). The differences in the methylation status of this CpG island are shown in detail in Figure 2A. The endometriotic stromal cells that express high levels of SF-1 showed a dense methylation pattern in this intron region of the *SF-1* gene. In contrast, the majority of the CpG sites were not methylated in SF-1-negative endometrial stromal cells. A significant difference was found in the methylation status between the 2 groups of cells ($P < .001$; Figure 2B).

The other 2 parts showed no differences in the methylation status between the 2 groups (assay 1: 185-bp region spanning 14 CpGs; assay 3: 577-bp spanning 70 CpGs; data were shown in Supplemental Figures 1–3, available online at <http://rs.sagepub.com/supplemental>).

Correlation Between *SF-1* mRNA Levels and Percentage Methylation

Previously, we reported strikingly higher mRNA levels of the *SF-1* gene in endometriotic stromal cells than that in endometrial stromal cells.¹¹ To characterize the effect of methylation changes in this intron region on *SF-1* gene expression, we determined the correlation between SF-1 mRNA levels and percentage methylation of the intron 1 region (assay 2) in the endometriotic stromal cells. As shown in Figure 3, a strong correlation was observed between RNA expression and percentage methylation (Spearman correlation coefficient = .96; $P < .001$).

Discussion

Genome-wide methylation studies have markedly changed the concept of tissue-specific differentially methylated regions (TDMRs) in the mammalian genome. A large body of literature has shown that DNA methylation at the promoter region is inversely correlated with transcription.^{15–19} We published a study that

links methylation and SF-1 mRNA expression in endometrial and endometriotic cells. In normal endometrial cells that do not express SF-1, the proximal promoter was found to be hypermethylated; whereas in endometriotic cells, which do express SF-1, the same region was hypomethylated.¹¹ Hoivik et al also reported that this region was hypomethylated in mouse and human cells that express SF-1, but hypermethylated in SF-1-nonexpressing cells. Their study also reported that the transcriptional activator present upstream of stimulatory factor 2 and RNA polymerase II was specifically recruited to this DNA region in cells in which the proximal promoter is hypomethylated, providing further support for the speculation that lack of methylation corresponds to transcriptionally active genes.²⁰ Thus, together, these studies demonstrate that methylation of the proximal promoter directs SF-1 expression in both normal and diseased tissues exhibiting aberrant levels of SF-1 mRNA.

Many recent studies using restriction landmark genomic scanning reported that developmentally related genes are overrepresented in TDMRs. Moreover, although some of the TDMRs are located in promoter regions, many functionally relevant binding sites for transcription factors probably exist in regions outside of gene promoters, particularly in introns.^{21–24} We found evidence for this in our previous study, where we reported a positive association between increased methylation of the CpG island at the exon 2/intron 3 region and SF-1 expression in endometriotic tissue.¹² Intriguingly, hypermethylation of this exon/intron region activates SF-1 mRNA expression in endometriotic cells; however, this region is distinct from the 5' promoter sequence, hypermethylation of which classically silences gene expression.¹² Hoivik et al also found that the DNA methylation status of the enhancers strongly correlates with their activity: They were demethylated in the CpG island of intron 4 to intron 6 in human or mouse cells where they were active but methylated in other SF-1/Ad4BP-expressing cells.²⁵ Shirohzu et al demonstrated that the upstream intron 1 CpG island sequence of mouse SF-1 was differentially methylated between an in vitro mouse cell culture system and normal mouse tissues; moreover, hypermethylation of this intron region activated SF-1 mRNA expression in adrenocortical Y-1 cells, but the methylation status of this region did not predominantly affect the tissue- or development-specific expression patterns of SF-1.²⁶

To gain further insights into the mechanisms that underlie the confined activity of the intronic enhancers and thus the tissue-specific expression of the SF-1 locus, we applied the CpGPlot identification analysis to the human SF-1 intron region (Figure 1A) and found a novel downstream CpG island around intron 1. Our data showed that the methylation status between the +3462 bp and +3633 bp regions of the *SF-1* gene was positively associated with mRNA levels in endometriotic cells.

Also, our results are consistent with recent studies and our previous reports indicate that methylation outside the promoter leads to increased gene expression.^{12,25,27–29} The ventromedial hypothalamic nucleus-specific enhancer that located in intron 6 was found to be hypermethylated in SF-1/Ad4BP-expressing cell lines from adrenal (Y1) and testis (MA10 and MSC-1).²⁵ Figure 4 summarizes what is currently known from our studies

Figure 4. Proposed mechanism for the regulation of steroidogenic factor I (SF-1) expression by DNA methylation of CpG islands in the promoter, intron 1, and exon 2 to intron 3 regions in eutopic endometrial and endometriotic cells. The open boxes (exon 1 and part of exon 2) indicate the noncoding exonic sequences. The dark boxes indicate the coding exonic sequences. DNA hypermethylation of the promoter CpG island and hypomethylation in the 2 intron regions exert inhibitory effects on SF-1 transcription in the endometrium (panel B), whereas hypomethylation of the promoter CpG island and hypermethylation of the 2 intron regions promote SF-1 transcription in endometriotic tissue (panel A).

about the epigenetic control of the *SF-1* gene in endometriosis. We have shown in this figure the proposed mechanism for the regulation of SF-1 expression by DNA methylation of CpG islands in the promoter, intron 1, and exon 2/intron 3 regions in eutopic endometrial and endometriotic cells^{11,12} (we have added the results of the current study too). The other CpG islands in the intron 6 and 3' downstream region of *SF-1* gene need to be evaluated in endometriosis in the future.³⁰ We have observed that pharmacologic demethylation leads to an overall increase in SF-1 mRNA expression, suggesting that the promoter methylation pattern may outweigh intronic modifications.

Taken together, these findings enhance our understanding of the mechanisms underlying SF-1 expression patterns in endometriotic stromal cells, which control the expression of steroid-metabolizing enzymes and subsequently the production of specific steroid hormones. The novel significance of this CpG island in regulating SF-1 expression can potentially be applied to other tissues expressing SF-1, such as the ovary, adrenal cortex, and the ventromedial hypothalamic nucleus.

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Authors' Note

The supplemental figures are available online at <http://rs.sagepub.com/supplemental>.

Declaration of Conflicting Interests

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