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Hypomethylation-induced expression of *S100A4* in endometrial carcinoma

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Expression of various *S100* genes has been associated with clinically aggressive subtypes in a variety of different cancers. We hypothesized that *S100A4* would be overexpressed in endometrial carcinoma compared to benign endometrium. Quantitative real-time RT-PCR (qRT-PCR) was used to quantify the mRNA level of *S100A4* in benign endometrium ($n=19$), endometrioid adenocarcinoma ($n=87$), and non-endometrioid tumors ($n=21$). Immunohistochemistry was used to verify the results of qRT-PCR and to assess protein localization. Possible mechanisms of *S100A4* gene regulation were also examined. *S100A4* was overexpressed in the grade 3 endometrioid tumors, uterine papillary serous carcinoma, and uterine malignant mixed müllerian tumor. Expression in grade 1 and grade 2 endometrioid tumors was comparable to that of normal endometrium, which was quite low. Expression was significantly higher in stage III and IV tumors compared with stage I. By immunohistochemistry, S100A4 was expressed in the tumor cell cytoplasm of poorly differentiated tumors, but was not detected in normal endometrial glandular epithelium. In benign endometrium, S100A4 expression was confined to stromal cells. *S100A4* was not regulated by estrogen or progesterone, and its expression in tumors was not significantly correlated to *estrogen receptor* or *progesterone receptor* content. However, methylation of the *S100A4* gene was detected in benign endometrium and grade 1 tumors with low *S100A4* expression. In contrast, grade 3 endometrioid tumors with high *S100A4* mRNA and protein expression showed no methylation of the gene. These methylation results were verified in endometrial cancer cell lines with differential baseline levels of S100A4 protein. These results suggest that hypomethylation is an important mechanism of regulating the expression of the *S100A4* gene. These results support the emerging concept that hypomethylation may play a role in the upregulation of genes during later stages of tumorigenesis.
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Endometrial carcinoma is the most common malignant neoplasm of the female genital tract and the fourth most frequently diagnosed cancer in women, following cancers of the breast, lung, and colon.[1] On the basis of histological and clinical features, endometrial carcinoma has been classified into two types.[2–4] Type I tumors (approximately 80% of all endometrial carcinomas) are typically low-grade (grade 1 or grade 2) endometrioid adenocarcinoma, most of which are confined to the uterus and have a favorable prognosis. Type II tumors (approximately 20%) are comprised of uterine papillary serous carcinoma, malignant mixed müllerian tumors, and clear cell carcinoma. The nature of high-grade (grade 3) endometrioid adenocarcinoma is more controversial, as a subset may behave more like the Type II tumors. Type II cancers are associated with extra-uterine spread and carry a high mortality rate. Previous studies have documented interesting molecular differences between endometrioid and non-endometrioid endometrial carcinomas.[5–8] Metastatic or recurrent endometrial carcinomas represent a significant treatment problem, as current chemotherapeutic and hormonal strategies are entirely ineffective. Our laboratory has a long-term goal of identifying novel molecular events important in advanced endometrial carcinoma, with the ultimate goal of designing rational, targeted therapies for extra-uterine disease.

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S100A4 is a member of a family of *S100* genes that encode calcium-binding proteins of the EF-hand type.[9,10] The cellular function of S100A4 protein is not known. In humans, an increase in *S100A4* expression has been correlated with aggressiveness and worse prognosis for patients with different types of carcinoma, including colorectal, gallbladder, pancreas, bladder, gastric, breast, non-small cell lung, and prostate cancers.[11–18] The exact mechanism for the metastasis-promoting activity by S100A4 is poorly understood. S100A4 may influence growth regulation, remodeling of the extracellular matrix, angiogenesis, cell detachment, and cell motility.[19] Recently, it has been shown that S100A4 promotes cellular motility via direct interaction with myosin-IIA.[20]

We hypothesized that *S100A4* is overexpressed in endometrial carcinoma compared to benign endometrium. In the present study, we quantified the differential expression of *S100A4* in benign endometrium and various histotypes of endometrial carcinoma. In addition, we explored possible mechanisms of regulation of this gene in normal endometrium and in endometrial carcinomas.

Materials and methods

Human Normal Endometrial Biopsies, Tumor Samples, and Cell Lines

This study was approved by the University of Texas MD Anderson Cancer Center Institutional Review Board (LAB01-718). Fresh frozen endometrial carcinoma specimens ($n=108$) and benign endometrial tissues ($n=19$) were obtained as residual tissues from hysterectomy surgical specimens submitted to the Department of Pathology, MD Anderson Cancer Center. A gynecological pathologist (RR Broaddus) microscopically reviewed H&E-stained slides to confirm surgical stage, tumor grade, and histotype, based on the criteria established by the International Federation of Gynecology and Obstetrics.[21]

Gene expression was analyzed in human endometrial biopsies from two different clinical trials involving hormonal therapy. In the first group, described previously,[7] healthy, postmenopausal women received Premarin (Wyeth Research), 0.625 mg/day p.o., for 3 months. In the second group, healthy, premenopausal women received depot medroxyprogesterone acetate (depot MPA; Pharmacia & Upjohn), 150 mg i.m. For both groups, baseline (pretreatment) and 3 month post-treatment endometrial biopsies were obtained, and a portion of each biopsy was frozen. For the depot MPA group, both the baseline and post-treatment endometrial biopsies were timed to be conducted on days 8 or 9 after the commencement of the menstrual cycle, such that the endometrium was in the proliferative phase. This was verified by microscopic examination of the portion of the endometrial biopsy that was formalin-fixed and paraffin-embedded.

The human endometrial adenocarcinoma cell lines HEC-1A, HEC-1B, and KLE were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Ishikawa cells were kindly provided by Changping Zou, PhD, University of Arizona. HEC-1B and Ishikawa cells were cultured in MEM with 10% FBS. HEC-1A and KLE cells were cultured in McCoy's 5a medium with 10% FBS and DMEM/F-12 with 10% FBS, respectively. All cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

RNA Isolation and Quantitative Real-Time RT-PCR

RNA was isolated from frozen tissue samples using TRIzol (Invitrogen, Carlsbad, CA, USA), followed by an additional purification step using the RNeasy kit (Qiagen, Valencia, CA, USA) following the manufacturer's recommendations.

Quantitative real-time RT-PCR (qRT-PCR) was performed utilizing the 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA) as previously described.[22] Probe-based real-time quantitative assays for *S100A4*, *estrogen receptor α* (*ER-α*), *estrogen receptor β* (*ER-β*), *progesterone receptor* (*PR*), and *18S rRNA* were developed using Primer Express software (Applied Biosystems) based on sequences from GenBank. The assays were developed and all qRT-PCR reactions were run at the Quantitative Genomics Core Laboratory (UT-Houston Medical School, Houston, TX, USA). The primer and probe sequences, accession number, and pertinent information for each assay are listed in Table 1. Each qRT-PCR experiment was performed in duplicate using assay-specific sDNAs (synthetic amplicon oligonucleotides, Biosource, Camarillo, CA, USA) serially diluted in 10-fold decrements to obtain a standard curve covering a 5-log range in template concentration. A linear relationship between the threshold cycle (C_t) and the log of the starting sDNA copy number was always established (correlation coefficient >0.99) and used to construct a standard curve. The copy number for each transcript assayed was interpolated from the standard curve using the ABI SDS software. The final transcript values were normalized to those determined for *18S rRNA* in a 1/500 dilution of the total RNA and are presented as molecules of transcript/molecules of *18S rRNA*.

Immunohistochemistry

Formalin-fixed, paraffin-embedded blocks of endometrial cancer and normal endometrium were retrieved from the archives of the Department of Pathology at MD Anderson Cancer Center. After initial deparaffinization, endogenous peroxidase activity was blocked with 0.3% H_2O_2. Deparaffinized sections were microwaved in 10 mmol/l citrate buffer (pH 6.0). The slides were then incubated for 1 h at room temperature using rabbit

Table 1 Probes and primers for qRT-PCR assays

Transcript	*Taqman primers and probe*	*Accession number*
18S rRNA	(535+) GAGGGAGCCTGAGAAACGG (602−) GTCGGGAGTGGGTAATTTGC (555+) FAM-TACCACATCCAAGGAAGGCAGCAGG-BHQ1 Lowest quantifiable level = 210 molecules; average assay efficiency = 104%	M10098
S100A4	69(+) CATGGCGTGCCCTCTG 133(−) TGCCCGAGTACTTGTGGAAG 89(+) FAM-AGGCCCTGGATGTGATGGTGTCC-BHQ1 Lowest quantifiable level = 210 molecules; average assay efficiency = 98%	NM_002961
ER-α	1394(+) TACTGACCAACCTGGCAGACAG 1490(−) TGGACCTGATCATGGAGGGT 1466(−) FAM-TCCACAAAGCCTGGCACCCTCTTC-BHQ1 Lowest quantifiable level = 150 molecules; average assay efficiency = 97%	NM_000125
ER-β	1317(+) AGTTGGCCGACAAGGAGTTG 1404(−) CGCACTTGGTCGAACAGG 1385(−) FAM-TGAGCTCCACAAAGCCGGGAATCT-BHQ1 Lowest quantifiable level = 170 molecules; average assay efficiency = 98%	NM_001437
PR	2689(+) GAGCACTGGATGCTGTTGCT 2754(−) GGCTTAGGGCTTGGCTTTC 2710(+) FAM-TCCCACAGCCATTGGGCGTTC-BHQ1 Lowest quantifiable level = 220 molecules; average assay efficiency = 99%	AF016381

ER-α, *estrogen receptor α*; *ER-β*, *estrogen receptor β*; *PR*, *progesterone receptor*; qRT-PCR, quantitative real-time RT-PCR.

polyclonal anti-human S100A4 (1:50; DAKO, Carpinteria, CA, USA), followed by biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) for 30 min, and finally with ABComplex (Vector Laboratories, Burlingame, CA, USA). For S100A4, the bound complex was visualized with 0.125% Amino-ethyl carbazole (AEC, Sigma, St Louis, MO, USA), 0.003% (v/v) H_2O_2. The sections were then counterstained in Mayer's hematoxylin. For negative controls, the primary antibody was replaced with PBS.

Western Blot Analysis

Endometrial cancer cell line lysates were prepared in ice-cold lysis buffer (50 mmol/l Tris (pH 8.0), 100 mmol/l NaCl, 0.1% SDS, 1% NP-40, 0.5 mM EDTA) containing the protease inhibitor cocktail Complete (Roche, Mannheim, Germany). Proteins (20 μg) were boiled for 5 min and then run on 15% SDS-PAGE gels. Samples were then blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), blocked in 5% skim milk in PBS, and probed with rabbit anti-human S100A4 antibody (1:200 dilution, DAKO, Carpinteria, CA, USA) or mouse anti-human β-actin antibody (1:1500 dilution, Sigma Chemicals, St Louis, MO, USA), followed by anti-rabbit or anti-mouse IgG secondary antibodies (Amersham Bioscience, Piscataway, NJ, USA). The Enhanced Chemiluminescence kit (Amersham Bioscience, Piscataway, NJ, USA) was used for detection.

S100A4 Methylation Assays

Samples of genomic DNA isolated from endometrial cancer cell lines and formalin-fixed, paraffin-embedded tissue sections of endometrial carcinoma were modified by sodium bisulfite as described by Herman *et al*.[23] Benign endometrial glandular epithelial cells were isolated from paraffin-embedded tissue sections using laser-capture microdissection (Leica Microsystems, Bannockburn, IL, USA). Endometrial cancer cells in paraffin-embedded sections were manually dissected from adjacent stroma using a 2.0 mm biopsy punch (Fray Products Corp., Buffalo, NY, USA). The methylation status of *S100A4* was determined by methylation-specific PCR as described by Herman *et al*,[23] which takes advantage of DNA sequence differences between methylated and unmethylated alleles after bisulfite modification. The sequence differences of bisulfite-treated DNA were detected by PCR using primers specific for either the methylated or unmethylated DNA sequences. Primer sequences for *S100A4* were kindly provided by Dr Michael Goggins and Dr Norihiro Sato, Johns Hopkins University[24,25] (*S100A4*-M forward, 5′-TAT ACGTTGTTGTTATAGTACG-3′ and *S100A4*-M reverse, 5′-ACT TCCTACTCCCGAATACG-3′; *S100A4*-U forward, 5′-ATATGTTGTTGTTATAGT ATGTG-3′ and *S100A4*-U reverse, 5′-CTTCCTACTCCCAAATA CAC-3′).

To determine if *S100A4* methylation and expression could be manipulated *in vitro*, KLE cells were exposed continuously to 1 μM 5-Aza-2′-deoxycytidine (5Aza-dC; Sigma, St Louis, MO, USA) for 4

days. RNA and DNA were then collected for qRT-PCR and methylation-specific PCR.

Statistical Analysis

Statistical differences were calculated using the Mann–Whitney *U*-test and ANOVA test. The Tukey test was performed for multiple comparisons. Correlation between two variables was evaluated by the Spearman rank correlation test. For analyses comparing baseline to 3 month post-treatment results, the paired sample *t*-test was used. Differences were considered significant if $P<0.05$.

Results

Overexpression of S100A4 in Endometrial Cancer

Using qRT-PCR, we quantified the transcript levels of *S100A4* in a series of endometrial endometrioid adenocarcinomas ($n=87$), uterine papillary serous carcinoma ($n=10$), uterine malignant mixed müllerian tumor ($n=11$), and benign endometrial tissues ($n=19$). *S100A4* message was significantly higher in the grade 3 endometrioid tumors, uterine papillary serous carcinomas, and uterine malignant mixed müllerian tumors compared with endometrioid tumors grades 1 and 2 and benign endometrium (Figure 1a). Interestingly, *S100A4* expression in the grades 1 and 2 tumors was not significantly elevated compared with benign endometrium (Figure 1a), implying a selective overexpression in the more poorly differentiated, high-grade tumors. *S100A4* expression was significantly higher in stage III and IV tumors compared with stage I tumors (Figure 1b).

Immunohistochemistry was performed to qualitatively confirm the results of qRT-PCR, and to characterize the localization of S100A4 protein in normal endometrium and endometrial cancer tissues. In all normal endometrial tissues, there was weak S100A4 expression limited to the cytoplasm of cells of mesenchymal origin, such as endometrial stromal cells, lymphocytes, and vascular structures. Glandular epithelial cells of benign endometrium did not express S100A4 (Figure 2a). Thus, it appears that the low-level expression of *S100A4* detected in normal endometrium in the qPT-PCR assay (Figure 1a) is due mainly to expression in stromal cells. Tumor cells in grade 1 tumors showed no S100A4 staining (Figure 2b), but immunohistochemical expression was high in grade 3 tumors (Figure 2c), uterine papillary serous carcinoma (Figure 2d), and uterine malignant mixed müllerian tumor (data not shown).

S100A4 Expression in Relationship to Exogenous Hormones and Steroid Hormone Receptors

To determine if *S100A4* was induced by either estrogen or progesterone, transcript expression was analyzed from baseline and 3 month post-treatment endometrial biopsies from women receiving either Premarin ($n=9$) or depot MPA ($n=5$). In a previous study examining the effect of Premarin on the endometrial tissue expression of retinoic acid metabolizing enzymes, retinoid receptors, and retinoic acid biomarker genes, we demonstrated that Premarin caused a 2- to 4-fold induction of *RALDH2*, *RARα*, *CRABP2*, and *tTG*. These changes in gene expression were significantly greater than the tissue expression at baseline, before Premarin treatment.[26] Similarly, we have previously reported that Premarin treatment significantly induced the endometrial tissue expression of the novel estrogen-regulated gene, *EIG121*.[7] In contrast, Premarin and depot MPA caused only slight differences in the endometrial tissue expression of *S100A4* compared with baseline; these differences were not statistically significant (Figure 3). Similarly, *S100A4* expression does not show differential expression in the estrogen-dominant proliferative phase endometrium compared with the progesterone-dominant secretory endometrium (data not shown). Therefore, in the endometrium, *S100A4* does not appear to be significantly regulated by estrogen or progesterone.

Historically, decreased estrogen receptor and progesterone receptor have been closely associated with higher endometrioid tumor grade.[4,27] Because elevated *S100A4* was only present in grade 3 tumors (Figure 1), we next wanted to determine if there was an association between increased *S100A4* transcript levels and decreased steroid hormone receptor expression. As expected, in the endometrioid tumors, the expression of *ER-α* and *PR* were positively and significantly correlated with each other (Table 2). However, *S100A4* expression was not strongly correlated to the expression of any of the hormone receptors examined (Table 2). Therefore, for endometrial carcinoma, it appears that expression of *S100A4* is independent of *ER-α*, *ER-β*, or *PR* expression.

Methylation Status of S100A4

The mechanism responsible for the regulation of *S100A4* gene expression is largely unknown. Previous studies suggested a possible role of DNA methylation in the control of tissue-specific expression of *S100A4*.[28] The *S100A4* promoter does not contain CpG islands, and CpG sites throughout the gene are sparsely distributed.[28,29] DNA methylation-dependent repression of several other genes lacking CpG-rich promoters has been previously described.[30,31] There is a correlation between hypomethylation of the CpG sites in the first intron of *S100A4* and gene activation in human lymphoma,[32] colon cancer cell lines,[32,33] and pancreatic cancer cell lines and primary pancreatic ductal adenocarcinomas.[24,25] Based on those previous findings, we analyzed the methylation status in the first intron of

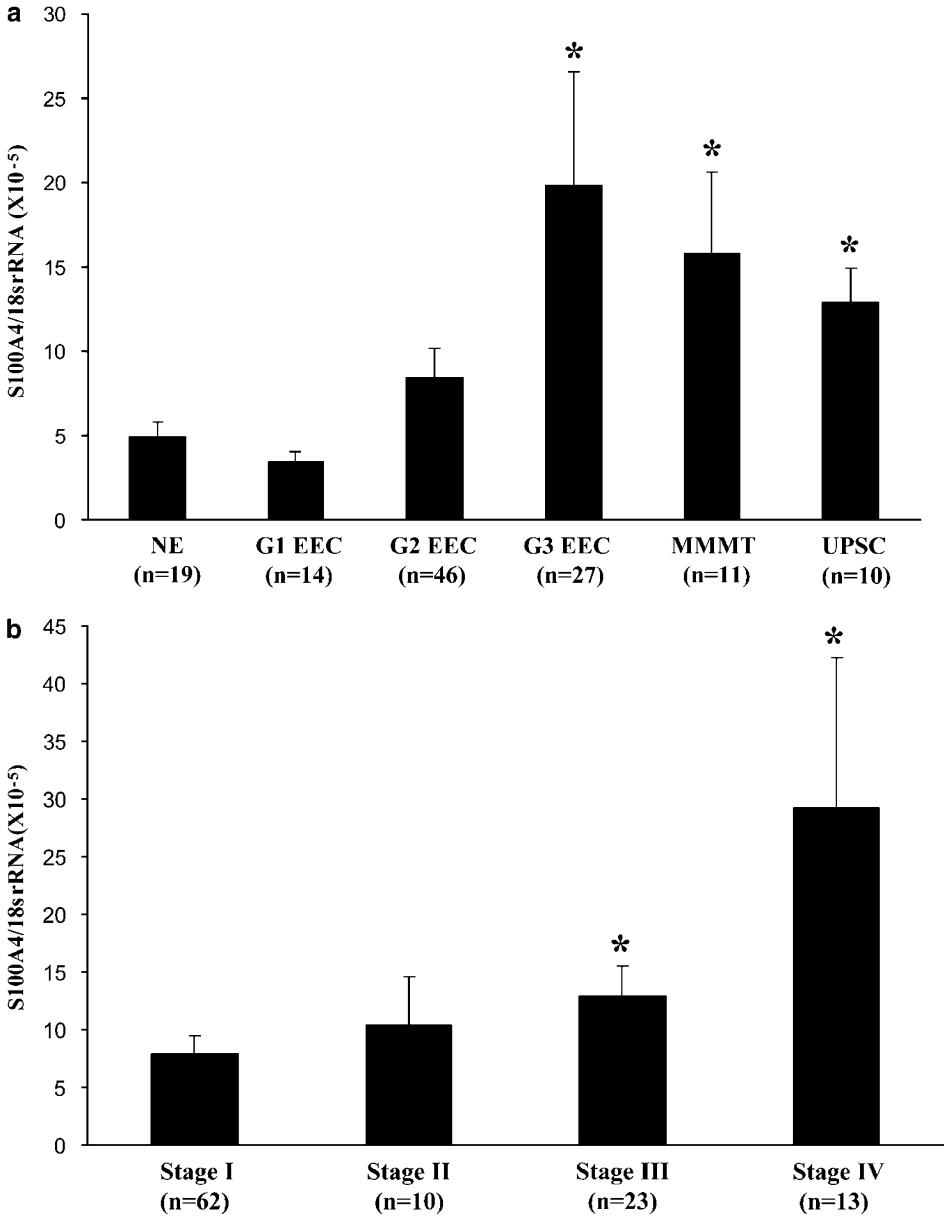

Figure 1 qRT-PCR analysis of *S100A4* in benign endometrium and endometrial carcinoma. *S100A4* transcripts were measured by qRT-PCR in normal, benign endometrium (NE; $n=19$) and various histotypes of endometrial carcinoma ($n=108$). Final transcript data were normalized to *18S rRNA* and are presented as the molecules of transcript/molecules of *18S rRNA*. Values shown are mean $\pm$ s.e. (**a**) *S100A4* was overexpressed in grade 3 endometrial endometrioid adenocarcinoma (EEC), uterine papillary serous carcinoma (UPSC), and malignant mixed müllerian tumor (MMMT) compared with grade 1 and 2 endometrial endometrioid adenocarcinoma and benign endometrium (*$P<0.01$). (**b**) *S100A4* was significantly overexpressed in stage III and IV tumors compared with stage I tumors (*$P<0.01$).

S100A4 using methylation-specific PCR. We detected methylated DNA in normal endometrial epithelial cells and grade 1 tumor cells with low *S100A4* expression (Figure 4). In contrast, grade 3 tumors with high *S100A4* expression showed no methylation of the gene (Figure 4). These results suggest that hypomethylation of the *S100A4* gene in grade 3 endometrioid tumors is associated with increased expression of *S100A4*. To confirm these findings, we next analyzed endogenous *S100A4* expression by qRT-PCR and Western blot and methylation status in several different endometrial carcinoma cell lines. S100A4 protein was expressed at different levels in the four cell lines tested, and there was a good concordance between *S100A4* mRNA and protein levels (Figure 5a and b). Because the KLE cells had very low endogenous expression of *S100A4* mRNA and protein, we examined the *S100A4* methylation status of these cells. At baseline, KLE cells demonstrate methylation of the *S100A4* gene (Figure 5c), similar to that seen in benign endometrium and grade 1 endometrioid tumors. Methylation of *S100A4* is not present in HEC-1A cells, which have high endogenous

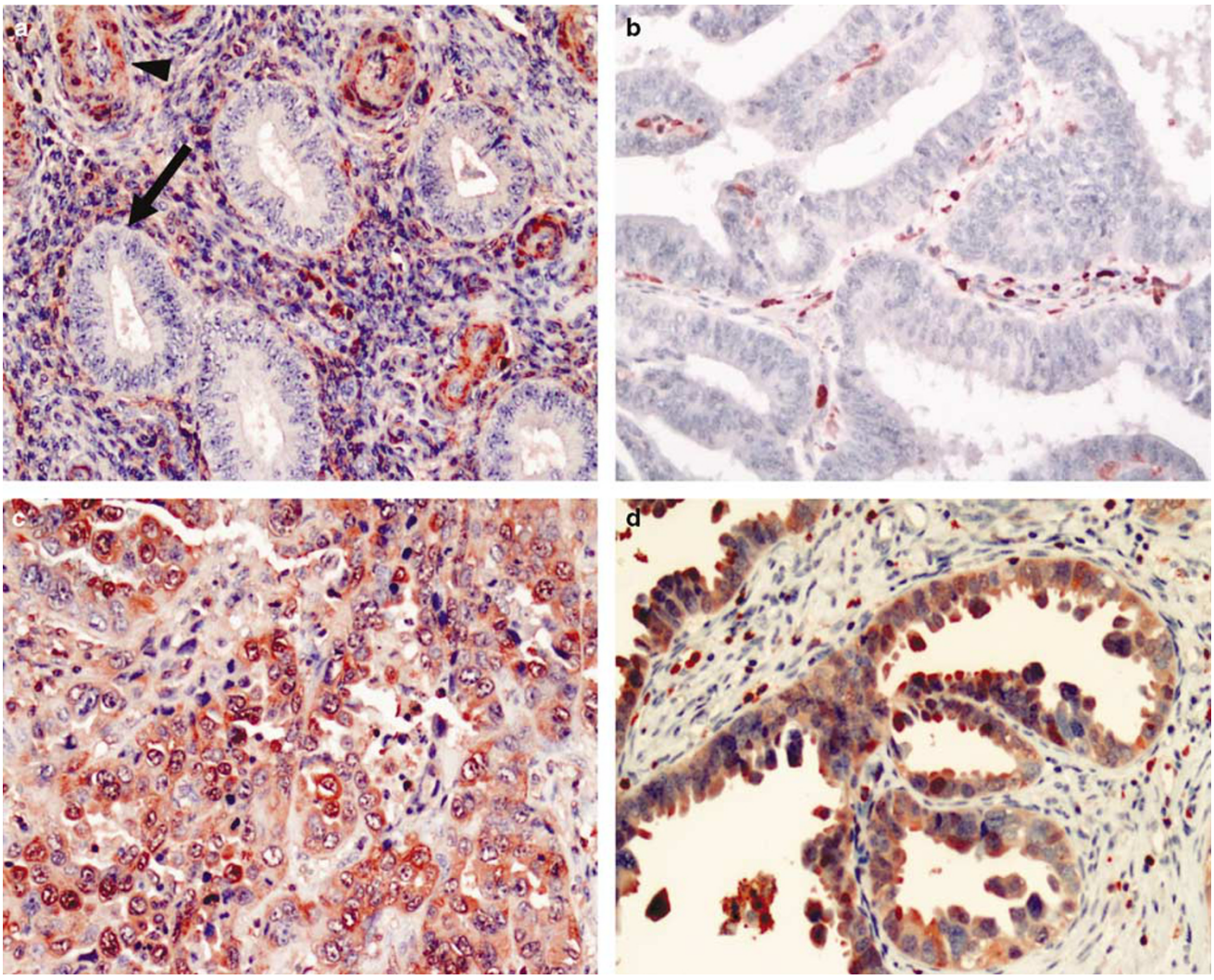

Figure 2 Immunohistochemical analysis of S100A4 protein expression. The immunohistochemical expression of S100A4 is demonstrated in benign endometrium (**a**), grade 1 endometrioid adenocarcinoma (**b**), grade 3 endometrioid adenocarcinoma (**c**), and uterine papillary serous carcinoma (**d**). S100A4 expression was absent in normal endometrial glandular epithelial cells (arrow), but present in stromal cells and vascular structures (arrowhead) (**a**). Similar to benign endometrium, well-differentiated grade 1 tumor cells did not express S100A4 (**b**). In contrast, S100A4 was strongly expressed in tumor cell cytoplasm for grade 3 tumors (**c**) and uterine papillary serous carcinoma (**d**). S100A4 protein was also strongly expressed in malignant mixed müllerian tumors (data not shown). All photomicrographs $\times 200$.

levels of *S100A4* mRNA and protein (Figure 5c). Treatment of the KLE cell line with 5-Aza-dC, an inhibitor of eukaryotic DNA methyltransferase, resulted in demethylation of *S100A4* and concomitant *S100A4* expression (Figure 5d and e). Thus, these results provide evidence that in the endometrium, DNA methylation is an important mechanism for regulating *S100A4* expression.

Discussion

Loss of steroid receptors and steroid hormone dependency has been a traditional indicator of aggressive disease in so-called 'hormone-dependent' cancers such as breast cancer and endometrial cancer.[4,27,34] Although previous studies suggested a correlation between S100A4 protein expression and loss of ER in breast carcinoma,[35] we did not observe a strong inverse correlation between *S100A4* expression and expression of the steroid hormone receptors *ER-α*, *ER-β*, and *PR* for endometrial cancer (Table 2). Furthermore, endometrial *S100A4* transcript levels were not significantly altered by either Premarin or depot MPA treatment (Figure 3). Interestingly, the expression level of *S100A4* in the grade 3 endometrioid tumors, uterine papillary serous carcinomas, and malignant mixed müllerian tumors was comparable to the expression of *ER-α* and *PR* in benign endometrium (data not shown). Therefore, in the higher-grade, poorly differentiated tumors, *S100A4* is abundantly expressed.

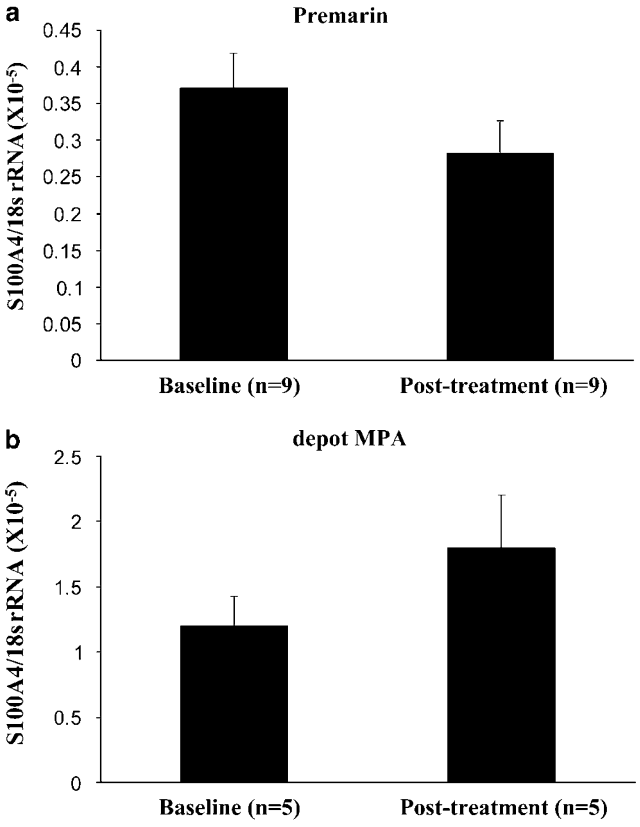

Figure 3 qRT-PCR analysis of *S100A4* in benign endometrium before and after exposure to estrogen or progesterone. Baseline and 3 month post-treatment endometrial biopsies were obtained from women receiving Premarin (**a**) or depot MPA (**b**). *S100A4* expression was quantified using qRT-PCR. *S100A4* expression was not significantly altered by Premarin ($P=0.187$) or depot MPA ($P=0.329$). Final transcript data were normalized to *18S rRNA* and are presented as the molecules of transcript/molecules of *18S rRNA*. Values shown are mean ± s.e.

Table 2 Correlation analysis between *S100A4* and steroid hormone receptors in endometrioid-type endometrial adenocarcinoma

	Correlation coefficient	P
ER-α vs PR	0.725	<0.001
S100A4 vs ER-α	−0.047	0.688
S100A4 vs ER-β	0.168	0.145
S100A4 vs PR	−0.23	0.056

ER-α, estrogen receptor α; ER-β, estrogen receptor β; PR, progesterone receptor.

A particularly striking finding from this study was the expression pattern of S100A4 protein in benign endometrium compared with various histotypes of endometrioid carcinoma. S100A4 was not expressed in the glandular epithelial cells of normal endometrium, but instead was present in cells of mesenchymal origin such as stromal cells, lymphocytes, and vascular structures (Figure 2). Similarly, S100A4 was not expressed in tumor epithelial cells of grade 1 carcinomas. However, S100A4 was overexpressed

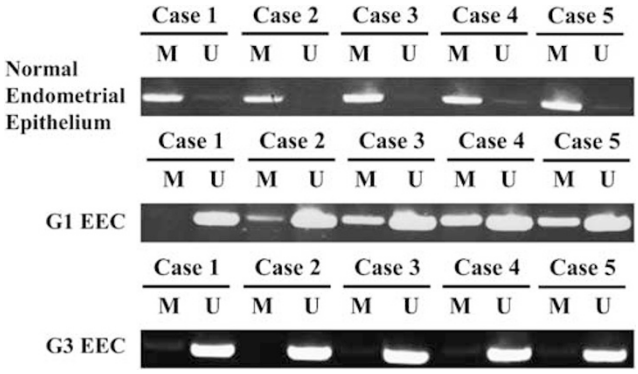

Figure 4 Methylation analysis of the *S100A4* gene. MSP was used to detect unmethylated (U) and methylated (M) DNA from the first intron of the *S100A4* gene in normal endometrial epithelial cells, grade 1 endometrioid tumors, and grade 3 endometrioid tumors ($n=5$ for each group). Methylated *S100A4* DNA was detected in 5/5 normal endometrial epithelial samples and 4/5 grade 1 tumors. No methylated *S100A4* DNA was detected in the grade 3 tumors (0/5).

in the tumor cell cytoplasm of grade 3 endometrioid carcinomas, uterine papillary serous carcinoma, and malignant mixed müllerian tumors (Figure 2). Such a differential expression pattern in benign endometrium (stromal cells only) compared with high-grade endometrial carcinoma (tumor epithelial cells) is suggestive of an epithelial-to-mesenchymal transition in gene expression pattern. Epithelial-to-mesenchymal transition has recently been recognized as a putative molecular mechanism underlying carcinoma invasion and metastasis.[36–38] During the process of epithelial-to-mesenchymal transition, epithelial cells actively downregulate cell–cell adhesion systems, lose their polarity, and acquire a mesenchymal phenotype with reduced intercellular interactions and increased migratory capacity.[36] The precise role of S100A4 is unknown, but there are several lines of evidence that S100A4 is a key mediator of EMT, not simply a 'bystander'. For example, in both cultured epithelial cells and a transgenic mouse model of metastatic breast cancer, *S100A4* expression was activated during EMT; antisense oligonucleotides suppressed *S100A4* expression and epithelial transformation.[39,40] Furthermore, recently it was shown that for colon carcinoma cell lines S100A4 was a direct target of the Wnt/β-catenin pathway. Specific knockdown of S100A4 in this system significantly reduced the migratory ability of these cells *in vitro*.[41]

Recently, several transcription factors that are molecular mediators of epithelial-to-mesenchymal transition, such as Twist, ZEB1, and HOXA10, have been shown to be expressed in endometrial carcinoma.[8,42,43]

Of particular interest is that ZEB1 has an expression pattern in endometrial carcinoma quite similar to that for S100A4. None of these transcription factors has been shown previously to directly regulate *S100A4* expression. Therefore, we investigated the possibility of epigenetic regulation of

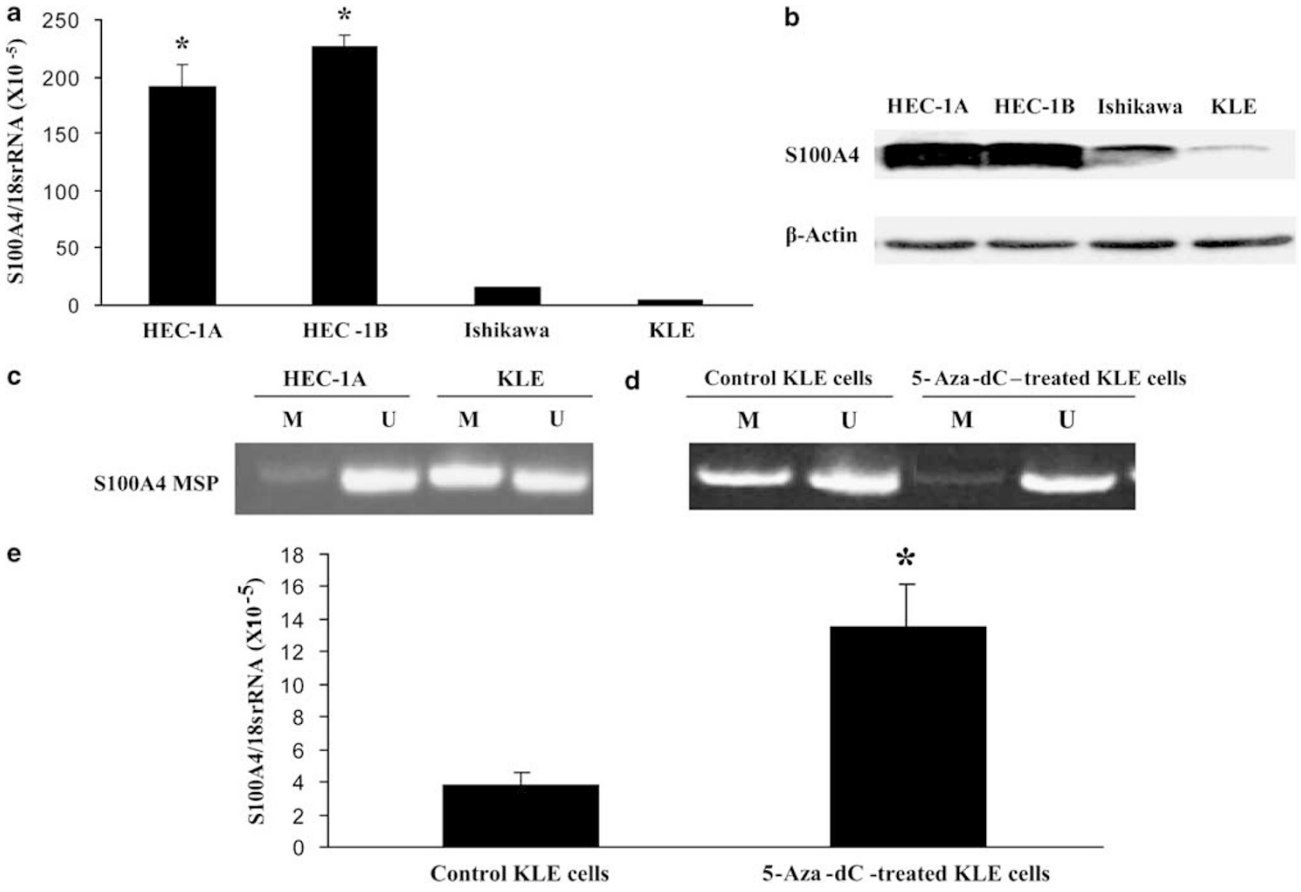

Figure 5 Correlation of *S100A4* expression with DNA methylation in human endometrial cancer cell lines. Basal expression of *S100A4* in EC cell lines by qRT-PCR (**a**) and Western blot (**b**). HEC-1A and HEC-1B cells had significantly higher *S100A4* gene expression than Ishikawa and KLE cells (*$P<0.001$). (**c, d**) Methylation-specific PCR analysis of *S100A4* gene methylation. At baseline, KLE cells have methylated *S100A4* DNA (**c**), which corresponds to the very low *S100A4* transcript and protein expressed by these cells. *S100A4* is unmethylated in HEC-1A cells (**c**), which has high endogenous *S100A4* expression. Following 5-Aza-dC treatment, very little methylated *S100A4* DNA is detected in KLE (**d**), and the transcript expression is significantly increased (**e**). For qRT-PCR experiments, the final transcript data were normalized to *18S rRNA* levels and are presented as the molecules of transcript/molecules of *18S rRNA*. Values shown are mean $\pm$ s.e. (*$P<0.05$).

S100A4 expression via methylation. In agreement with previous studies examining the role of DNA methylation in *S100A4* gene expression in colon and pancreatic cancers,[25,32,33] we found that overexpression of *S100A4* was associated with hypomethylation within the first intron of the *S100A4* gene in the high-grade endometrioid endometrial cancers (Figure 4). Increased expression of *S100A4* in KLE cells following 5Aza-dC treatment (Figure 5d and e) further supports the hypothesis that DNA hypomethylation induces *S100A4* gene expression in endometrial cancer. A hallmark of cancer is a paradoxical aberration of DNA methylation patterns, with a global loss of DNA methylation that coexists with regional hypermethylation of certain genes.[44] It has been proposed that hypermethylation and hypomethylation in cancer are independent processes, which target different programs at different stages in tumorigenesis.[45] Hypermethylation and silencing of genes that regulate proliferation are proposed to be critical for deregulated growth early in carcinogenesis, while hypomethylation and activation of other genes may be more important for metastasis.[45,46] The importance of hypermethylation and inactivation of tumor suppressor genes is well documented in various cancers, but the role of DNA hypomethylation in more advanced cancers and metastasis has been less studied and is still hypothetical. Accumulating data from the last several years have led to the hypothesis that hypomethylation plays a role in activating certain genes required for metastasis and invasion of a few cancer types other than endometrial cancer.[15,24,47] Results from our study in endometrial cancer add further evidence in favor of the idea that aberrant DNA hypomethylation contributes to the phenotype of more poorly differentiated carcinomas.

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