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S100A4 Mediates Endometrial Cancer Invasion and is a Target of TGF-β1 Signaling

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Abstract

The molecular mechanisms of endometrial cancer invasion are poorly understood. S100A4, also known as FSP1 (fibroblast specific protein 1), has long been known to be a molecular marker of fibrosis in a variety of different fibrotic diseases of the lungs, liver, kidney, and heart. We demonstrate here that increased expression of *S100A4* is associated with advanced stage endometrial cancer and decreased recurrence free survival. To verify the essential role of *S100A4* in invasiveness of endometrial cancer, *S100A4* expression was down-regulated by RNAi in HEC-1A cells, which resulted in undetectable S100A4 protein and significantly decreased migration and invasion. Due to the established connection between TGF-β1 and S100A4 induction in experimental models of kidney and liver fibrosis, we next examined whether TGF-β1 could also regulate S100A4 in endometrial cancer cells. TGF-β1 stimulated endometrial cancer cell migration and invasion with a concomitant increase in S100A4 protein. Induction of S100A4 was associated with the activation of Smads. TGF -β1 mediated endometrial cancer cell motility was inhibited by *S100A4* siRNA. In aggregate, these results suggest that S100A4 is a critical mediator of invasion in endometrial cancer and is upregulated by the TGF-β1 signaling pathway. These results also suggest that endometrial cancer cell invasion and fibrosis share common molecular mechanisms.

Keywords

endometrial cancer; S100A family genes; S100A4; cancer invasion; epithelial-to-mesenchymal transition

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Introduction

S100A4, also known as Fsp1 (fibroblast-specific protein 1), is well-known to be over-expressed in fibrotic diseases of the lung, kidney, liver, and heart1-5. In normal adult tissues, its expression is confined to fibroblasts and other cell types of mesenchymal origin. Previously, we identified high expression of S100A4 to be associated with adverse clinical-pathological factors for endometrial cancer, including higher endometrioid grade, non-endometrioid histology, and advanced stage6. In normal endometrium and lower grade endometrioid carcinomas, S100A4 protein is only weakly expressed in stromal cells, with no epithelial expression. However, in the higher grade endometrioid carcinomas and the non-endometrioid carcinomas, S100A4 protein is expressed in tumor epithelial cells6. Because of this drastic change in the pattern of S100A4 expression, we have speculated that epithelial-to-mesenchymal transition (EMT) is a critical event in the development of an invasive endometrial cancer.

S100A4 belongs to the *S100* gene family, a multi-gene family of Ca^{2+}-binding proteins of the EF-hand type. These genes are involved in a variety of cellular processes, such as immune response, differentiation, cytoskeleton dynamics, and cell growth7,8. To date, 20 members of the *S100* family have been identified in humans8. The majority of the *S100* genes are clustered in a region of chromosome 1q21, which is frequently rearranged in a number of malignancies, including endometrial cancer9. Members of the *S100* gene family are highly conserved, but individual S100 proteins show cell- and tissue-specific expression patterns7. Interestingly, several S100 proteins, such as S100A2, S100A4, S100A6, S100A7, S100A9, S100A10, and S100A11 are specifically up-regulated in aggressive, advanced, metastatic tumors relative to non-invasive, non-metastatic tumors6,10-18. The preferential expression patterns of these proteins in more invasive and metastatic tumors has led them to become regarded as potential prognostic markers8. In most cases the mechanisms of action of S100 proteins and the functional implication of their altered expression in cancers are still unknown. Furthermore, the mechanisms regulating expression of the *S100* genes in cancers are largely unknown. In this study, we examined the transcriptional expression of *S100A2*, *S100A4*, *S100A6*, *S100A7*, *S100A9*, *S100A10* and *S100A11* in a large set of human endometrial tumors and normal endometrial tissues and further correlated their expression with well-documented clinicopathologic parameters of aggressiveness and poor prognosis of endometrial cancer. Only *S100A4* expression was significantly associated with all of these aggressive features, so it was chosen for more detailed mechanistic studies which are summarized here.

Materials and Methods

Human normal endometrial tissues, tumor samples, and cell lines

This study was approved by the University of Texas M.D. Anderson Cancer Center Institutional Review Board (LAB01-718). Fresh frozen endometrial carcinoma specimens (n=108) and normal endometrial tissues (n=19) were obtained as residual tissues from hysterectomy surgical specimens submitted to the Department of Pathology, M.D. Anderson Cancer Center. The frozen tumor tissues were acquired from the luminal portion of the endometrial cancer so as not to interfere with pathological staging of myometrial invasion.

A gynecological pathologist (RRB) 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 Obstetrics19. Tumor recurrence and recurrence free and overall survival were identified by review of clinical documentation in the electronic medical record. A diagnosis of recurrent disease was made by identification of a new lesion on clinical exam or visualization of a new mass on radiographic imaging. Recurrence free survival was defined as the interval between the date of primary surgical treatment and the date of tumor recurrence, and overall survival as the time from primary surgery until date of death or date of last recorded follow-up.

The human endometrial adenocarcinoma cell lines HEC-1A and KLE were obtained from the American Type Culture Collection (ATCC, Rockville, MD). These endometrial cancer cell lines were chosen because we have previously shown that the HEC-1A cells have high endogenous levels of S100A4 and are highly invasive. The KLE cells, on the other hand, have low endogenous levels of S100A4 and are minimally invasive6. All endometrial cancer cells were cultured in McCoy's 5a medium with 10% FBS.

RNA isolation

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

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed utilizing the 7700 Sequence Detector (Applied Biosystems, Foster City, CA) as previously described 20. Probe-based real-time quantitative assays for *S100A2*, *S100A4*, *S100A6*, *S100A7*, *S100A9*, *S100A10*, *S100A11*, *Snail*, and 18S rRNA were developed using Primer Express software (Applied Biosystems) based on sequences from Genbank. The assays were developed and all real-time qRT-PCR reactions were run in the Quantitative Genomics Core Laboratory (UT-Houston Medical School, Houston, TX). 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) 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 (Ct) 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 by the ABI SDS software. The final transcript values were normalized to those determined for 18S rRNA.

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 minutes and then subjected to 15% or 10% SDS-PAGE gels. Samples were then blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA), blocked for one hour in 5% skim

milk in PBS, and probed with anti-S100A4 antibody (1:200 dilution, DAKO, Carpinteria, CA); anti-β-actin antibody (1:100,000 dilution, Sigma Chemicals, St Louis, MO), anti-α-Tubulin antibody (1:1000 dilution, Santa Cruz Biotechnology); anti-Phospho-Smad2 (Ser465/467) (1:1000 dilution, Cell Signaling Technology); anti-Smad2 (1:1000 dilution, BD Bioscience, Bedford, MA); anti-AKT (1:1000 dilution, Cell Signaling Technology, Danvers, MA); anti-phospho-AKT (Ser 473) (1:1000 dilution, Cell Signaling Technology); anti-Erk1/2 and anti-phospho (Thr-202/Tyr-204)-Erk1/2 (1:1000 dilution, Cell Signaling Technology), followed by anti-rabbit or anti-mouse IgG secondary antibodies (Amersham Bioscience, Piscataway, NJ). The Enhanced Chemiluminescence kit (Amersham Bioscience, Piscataway, NJ) was used for detection.

RNA interference experiments

S100A4 siRNA pool was commercially purchased from Dharmacon (Lafayette, CO). Negative non-targeting control siRNA from Dharmacon was also used. By using a DharmaFECT transfection kit (Lafayette, CO), cells were transfected with either non-targeting control siRNA (100nM) or *S100A4* siRNA (100 nM). *S100A4* shRNA construct was commercially purchased from Open Biosystems (Huntsville, AL). Transfections were performed using the Arrest-In transfection reagent from Open Biosystems (Huntsville, AL), and stable transfectants were created with the addition of 1 μg/mL puromycin (Sigma-Aldrich, St Louis, MO) to the culture medium. A non-silencing shRNA construct (Open Biosystems, Huntsville, AL) was used as a negative control. The effectiveness of gene silencing was determined by performing qRT-PCR and western blot analysis.

In vitro cell proliferation

HEC-1A or KLE endometrial cancer cells were seeded in triplicate in tissue culture dishes and cultured in complete medium. Medium was replaced every second day. Every 24 hours for 5 days, the growth medium was collected, cells were trypsinized and added to the growth media, centrifuged at 1000 RPM for 5 minutes, re-suspended in a known volume of media, and counted in trypan blue with a hemacytometer to determine the total number of viable cells present.

Migration and invasion assays

Migration and invasion assays for HEC-1A cells with manipulated S100A4 expression were performed as described by Albini et al. 21,22 In brief, HEC-1A cell migration was quantified using a modified two-chamber migration assay (8 μm membrane pore size, BD Bioscience, Bedford, MA) or a modified two-chamber invasion assay (membrane coated with a layer of Matrigel extracellular matrix proteins, BD Bioscience) according to the manufacturer's instructions. The bottom chamber was filled with 750 μL of NIH/3T3 conditioned media, and the insert was placed into the 5 conditioned media. An aliquot containing 2×10^5 cells suspended in serum free culture media was seeded in the top insert. NIH/3T3 conditioned medium served as a chemoattractant in the lower chamber. The HEC-1A cells were allowed to migrate/invade for 22 hours at 37 °C. Cells in the upper chamber were carefully removed using cotton buds, and cells attaching to the lower surface of the membrane were fixed and stained with the HEMA 3 Stain set (Fisher Diagnostics,

Middletown, VA). Quantification was performed by counting the stained cells under light microscopy.

Wound healing assays

Wound-healing assays were performed to confirm the Boyden chamber migration data. HEC-1A cells with manipulated S100A4 expression were grown in McCoy's 5A with 10% FBS in six-well cell culture plates until they reached 100% confluence. A small linear scratch was introduced in the middle of the confluent cells using a 10 μl pipette tip and a photomicrograph was taken for the 0 time point. All scratches were carefully performed to assure equal width of the scratch made and also as to the location of the scratch so that all photomicrographs after time 0 were taken at the same point of the scratch. Cells were then returned to the incubator for continued growth. Cells were only removed from the incubator at the designated time points (24 and 48 hours) when photomicrographs were taken to monitor the cell migration progress.

TGF-β1 treatments in vitro

HEC-1A cells or KLE cells were seeded at low density (10^5 cells/well) on 6-well plates (day 0). The cells were allowed to attach for 24 h, after which time they were rinsed twice with PBS, followed by the addition of serum-free medium containing either 2.5 ng/ml of human TGF-β1 (R & D systems, Minneapolis, MN) or an equal volume of TGF-β1 vehicle (4 mM HCl with 1 mg/mL bovine serum albumin) and incubated for a period of time as indicated. The medium, with growth factor or vehicle, was replaced daily. The cells were photographed and evaluated for morphological change every 48 hours. All cells were assayed in triplicate for both control and TGF-β1 treated conditions.

Statistical analysis

Statistical differences were calculated using the Mann-Whitney U test and ANOVA. The Tukey HSD procedure was used to adjust for multiple comparisons. Correlation between two variables was evaluated by the Spearman rank correlation test. The Kaplan-Meier method was applied to generate survival estimates, and Cox proportional hazards regression models were used to model the association between survival and covariates of interest. For all statistical analyses, differences were considered significant if $p<0.05$.

Results

Over-expression of S100A2, S100A4, S100A7, and S100A9 in endometrial cancer

Using real-time quantitative PCR (qRT-PCR), we quantified the transcript levels of *S100A2*, *S100A4*, *S100A6*, *S100A7*, *S100A9*, *S100A10*, and *S100A11* in a series of endometrial cancers (endometrioid tumors, n=71; non-endometrioid tumors, n=21) and normal endometrial tissues (n=17). These *S100* genes were chosen because they had previously been shown in the literature to be over-expressed in different cancer types. *S100A2*, *S100A4*, *S100A7*, and *S100A9* all showed elevated levels of mRNA in endometrial cancer compared to benign endometrium (Figure 1). For each of these *S100* genes, mRNA expression tended to be highest in the higher grade tumors. However, there were distinct differences in expression among the four genes. *S100A4* was over-expressed only in grade 3 endometrioid

carcinoma and the non-endometrioid tumors uterine papillary serous carcinoma (UPSC) and malignant mixed Mullerian tumor (MMMT); these histotypes are most often associated with deep myometrial invasion and metastasis. *S100A7*, on the other hand, was significantly elevated in grade 2 and grade 3 endometrioid carcinoma, but was not increased in the non-endometrioid tumors. Overall, *S100A7* expression was very low compared to the other *S100s* examined, so the significance of its over-expression in higher grade endometrioid tumors is uncertain. *S100A2* expression was significantly increased in all grades of endometrioid tumors as well as in the non-endometrioid tumors. Expression in UPSC, although elevated overall compared to benign endometrium, was highly variable. *S100A9* was significantly increased in grades 2 and 3 endometrioid tumors as well as in UPSC and MMMT. Mean expression values for *S100A6*, *S100A10*, and *S100A11* were not significantly different from normal endometrium for any of the tumor histotypes investigated (data not shown).

Expression of S100 genes in relation to clinicopathologic indicators of endometrial cancer invasiveness

We next compared the expression levels of the four *S100* genes that were elevated in endometrial cancers compared to benign endometrium in relation to well-established clinicopathologic indicators of endometrial cancer invasiveness, including myometrial invasion greater than 50% uterine wall thickness, lymphatic/vascular invasion, and extra-uterine disease (Table 2). Only over-expression of *S100A4* was significantly associated with all three of these indicators of endometrial cancer invasiveness. Importantly, *S100A4* was also significantly over-expressed in the histotypes of endometrial cancer (grade 3 endometrioid carcinoma, UPSC, and MMMT) most often associated with deep myometrial invasion and metastasis (Figure 1). Rates of recurrence, overall survival, and progression-free survival were analyzed in relation to *S100A4* expression. *S100A4* was significantly higher in the 27 primary tumors from patients who later had recurrence (15.51 vs 8.06, p=0.009). *S100A4* expression did not correlate with overall survival (HR 1.87 [95% CI 0.64-5.47]; p=0.25). However, there was significantly decreased recurrence free survival with increasing *S100A4* expression (HR 4.39 [95% CI 1.48-13.09]; p=0.001). Therefore, based on the results described above, we chose to study *S100A4* in greater detail. It is important to note, however, that other *S100* genes, especially *S100A2*, *S100A7*, and *S100A9*, may also play important roles in endometrial cancer.

Effects of S100A4 gene knockdown on cell migration and invasion and cell proliferation

Based on the important clinical associations of high S100A4 expression with depth of myometrial invasion, stage, and recurrence, we next wanted to test the hypothesis that overexpression of S100A4 is an actual contributor to the aggressive phenotype in endometrial cancer. To accomplish this, we manipulated the expression of S100A4 in endometrial cancer cell lines and determined its effects on cell migration and invasion. From our previous published studies, we have shown that HEC-1A and HEC-1B endometrial cancer cells have high endogenous S100A4 mRNA and protein levels and are highly invasive, while Ishikawa and KLE endometrial cancer cells have low levels of endogenous S100A4 and are minimally invasive6. So, we chose HEC-1A endometrial cancer cells for the siRNA experiments. Transient transfection of *S100A4* siRNA resulted in significant

down-regulation of both S100A4 mRNA and protein. At 36 hours post-transfection, the mRNA expression levels of *S100A4* decreased to roughly 5% of the non-targeting siRNA control expression levels (Figure 2A). Similarly, S100A4 protein expression was reduced dramatically at 48 hours post-transfection and the reduction persisted at 5 days (Figure 2B). In comparison with non-targeting siRNA transfected cells, cells at 48 hours post-transfection of *S100A4* siRNA exhibited a significant reduction in cell migration and invasion (Figure 2C, 2D). Thus, the results of these experiments suggest that S100A4 is a critical mediator of endometrial cancer cell migration and invasion.

For long-term stable suppressed expression of S100A4 in HEC-1A cells, stable *S100A4* knockdown clones were generated by transfection of *S100A4* specific short hairpin RNA (shRNA) into HEC-1A cells, and stable transfectants were selected and expanded. Two *S100A4* knockdown clones were produced, with undetectable S100A4 protein compared to control empty vector clones (Figure 3A). Stable *S100A4* gene knockdown caused significant decreases in cell migration in a wound assay (Figure 3B) and in migration and invasion in the two-chamber assay (data not shown), similar to what was observed with transient knockdown. For the endometrial cancer cells, knockdown of *S100A4* expression had no significant effect on cell proliferation *in vitro* (Figure 3C). In aggregate, results from our clinical-pathological associations and the more functional studies from the HEC-1A endometrial cancer cells provide strong evidence that S100A4 is an important molecular mediator of endometrial cancer invasion.

TGF-β1 signaling upregulates S100A4 expression

Activation of TGF-β and S100A4 have been shown to be important in models of kidney and liver fibrosis1-3. However, S100A4 has not been previously linked to TGF-β1 in any cancer system. Therefore, we were interested in determining whether TGF-β1 induces S100A4 in the human endometrial cancer cell line HEC-1A. We previously published that for this cell line, the *S100A4* gene was not methylated, which corresponded to high basal levels of S100A4 protein expression6. S100A4 mRNA and protein were up-regulated 96 hours after TGF-β1 treatment (Figure 4A, 4B). This late induction of S100A4 is consistent with other reports that S100A4 was induced by prolonged stimulation of TGF-β1 in cultured intrahepatic biliary epithelial cells or renal epithelial cells1-3. We have previously demonstrated that minimally invasive KLE endometrial cancer cells have low endogenous *S100A4* transcript and protein due to hypermethylation of the *S100A4* gene6. TGF-β1 does not induce *S100A4* message in the KLE cells (Figure 4C), suggesting that epigenetic modifications, such as *S100A4* gene methylation, may play an important role in determining responsiveness to TGF-β1.

S100A4 is required for TGF-β1 effects on cell migration and invasion

In contrast to the growth inhibitory effects of TGF-β1 on the early stages of carcinogenesis, TGF-β1 can also act as a promoter of tumor cell invasion and metastasis in the later stages of tumorigenesis 23. We first examined the effects of TGF- β1 on cell growth in HEC-1A cells. The growth of HEC-1A cells was inhibited 48 hours after TGF-β1 stimulation (data not shown). However, HEC-1A cells pre-treated with TGF-β1 for 5 days exhibited increased migratory capability and invasiveness (Figure 5A, 5B), concomitant with an increase in

S100A4 expression. We next wanted to determine if S100A4 was necessary for the invasion and migration induced by TGF- β1. In HEC-1A cells transfected with *S100A4* siRNA, TGF-β1 could not stimulate significant migration and invasion (Figure 5C, 5D). Consistent with our previous results (Figure 2), the *S100A4* siRNA also significantly decreased the basal capability of these cells to migrate and invade (Figure 5C, 5D). Based on these data, S100A4 expression is required for TGF-β1 to exert its pro-migratory and pro-invasive effects on endometrial cancer cells. Therefore, S100A4 is a critical effector of endometrial cancer cell invasiveness.

Interestingly, we also found that the addition of TGF-β1 to HEC-1A cells elicited morphological changes, as treated cells showed a somewhat elongated, spindle-like, fibroblast appearance compared with controls, which is indicative of epithelial-to-mesenchymal transition (EMT). EMT is classically associated with stimulation of cell motility, down-regulation of epithelial markers such as E-cadherin and cytokeratins, and up-regulation of mesenchymal markers such as vimentin and fibronectin. To determine whether TGF-β1 could induce EMT of HEC-1A cells *in vitro*, we then examined the expression of these EMT marker proteins. However, TGF- β1 treatment for 5 days did not alter the expression of any of these proteins (data not shown). Nevertheless, TGF-β1 did elicit cell morphological changes and enhanced migratory ability and invasiveness of HEC-1A cells *in vitro*, which has been recognized as cell scattering or incomplete EMT24. The TGF-β1 induced cell scattering phenotype has been reported previously in normal mammary epithelial NMuMG cells24-27.

TGF-β1 induces Smad2 activation

Up-regulation of S100A4 protein by extended TGF-β1 treatment suggested that *S100A4* is not one of the early response genes of TGF-β1 and that secondary or tertiary signaling pathways are likely needed to activate S100A4 expression. Next, we evaluated the TGF-β1 signaling pathways that are potentially associated with the cell scattering phenotype and upregulation of S100A4. Both Smad-dependent and Smad-independent (i.e. Akt-and Erk-dependent) TGF-β1 pathways have been implicated in EMT in various epithelial cell types28-35. First, the Smad-dependent signaling pathway was examined (Figure 6). The phosphorylation of Smad2 was examined at various time points after adding TGF-β1 to HEC-1A cells. TGF-β1 induced phosphorylation of Smad2 (pSmad2) at all the time points examined, and the level of Smad2 phosphorylation remained elevated for the duration of the experiment without affecting total Smad2 expression. In contrast, pSmad2 was not present in cells treated with vehicle control. Activation of Smad-independent pathways (phospho-Akt and phosphor-Erk1/2), however, was not observed following TGF-β1 treatment (data not shown). This suggested that Smad-independent TGF-β signaling was not significantly involved in mediating TGF-β1 effects on HEC-1A cells. Taken together, these data indicate sustained phosphorylation of Smad2 is associated with TGF-β1-enhanced cell migration and invasion and the up-regulation of S100A4 in HEC-1A cells.

Discussion

Increased levels of TGF-β1 are observed in many human tumors, including endometrial cancer, and are associated with poor clinical outcome36-42. TGF-β1 is also an important stimulator of fibrosis in kidney transplant rejection and liver cirrhosis; in these settings, TGF-β1 is known to induce S100A41-3. Since TGF-β1 enhanced cell motility and invasion in endometrial cancer cells, we tested the effect of TGF- β1 on S100A4 expression. We found that S100A4 was up-regulated following prolonged TGF-β1 treatment. Moreover, S100A4 was required for the increased cell motility and invasion of HEC-1A cells by TGF - β1, because in HEC-1A cells transfected with S100A4 siRNA, the effects of TGF- β1 on the basal migration and invasion rates were significantly reduced. We propose that in invasive endometrial cancers, S100A4 is a significant downstream effector of the TGF- β1 signaling pathway through which TGF-β1 exerts its pro-migratory and pro-invasive effects on endometrial cancer cells. Therefore, well-established molecular mediators of fibrosis (TGF-β and S100A4) are also important in mediating cancer cell migration and invasion.

One of the mechanisms for transcriptional regulation by DNA methylation involves the binding of methylated DNA binding proteins such as MeCP2 to methylated regions. MeCP2 then recruits transcription repressor complexes containing histone deacetylases to the methylated gene precipitating an inactive chromatin structure, blocking the association of transcription activators and leading to the silencing of gene expression43-45. This might explain the differential effects of TGF-β1 on HEC-1A and KLE endometrial cancer cells. We have also previously shown that the expression of HOXA10 in endometrial carcinoma is regulated by methylation46. Specifically, HOXA10 is over-expressed in low grade (grade 1) endometrioid carcinomas, but its expression is low in the more clinically aggressive grade 3 endometrioid carcinoma and uterine papillary serous carcinoma. This differential expression pattern is regulated by methylation of the *HOXA10* gene. Endometrial cancer cells with manipulated *HOXA10* expression have altered *in vitro* invasion characteristics. In aggregate, these results suggest that epigenetic modifications, such as gene methylation, can play a potentially important role in regulating endometrial cancer invasion.

Previously, *S100A4* gene suppression by siRNA led to reduced cell proliferation in a prostate cancer cell line47. However, S100A4 knockdown did not affect endometrial cancer cell proliferation *in vitro* in our current study. Our results are actually quite consistent with previous published findings that transgenic mice overexpressing S100A4 in the mammary epithelium are phenotypically indistinguishable from wild-type mice, demonstrating that S100A4 itself is not tumorigenic48. It is likely that tissue-specific factors dictate whether S100A4 has an effect on cell proliferation. Additionally, invasion is a complex process that involves several different steps of cell migration and proliferation. For endometrial cancer, cell migration and proliferation might represent independent processes that are regulated by different signal transduction pathways. Experimental evidence has shown the lower proliferation rate of migratory cells in comparison with the tumor core, which indicates an inverse correlation between mobility and cell proliferation49-51. Thus, in certain systems, proliferation and migration of tumor cells may be mutually exclusive phenotypes.

The exact mechanism by which S100A4 stimulates invasion is not understood. S100A4, which is primarily a cytoplasmic protein, could facilitate cancer cell movement by physical interaction with various effectors52. S100A4 protein can bind to cytoskeletal proteins such as actin53, tropomyosin54, and myosin-II55-57. The interaction of myosin-IIA and S100A4 provides a direct link between the actomyosin cytoskeleton and the modulation of cellular motility by S100A4. Localization studies show that during chemotaxis, both S100A4 and myosin-IIA localize primarily to the leading edge of forward cell protrusions58.

Additionally, it has been proposed that S100A4 promotes cell invasion by remodeling of the extracellular matrix52. Dysregulation of metalloproteinases is essential for the remodeling of extracellular matrix proteins and for tumor cell migration and invasion. Metastatic osteosarcoma cell lines with down-regulated S100A4 have a reduction of the mRNA levels of *MMP2*, *membrane-type 1-MMP*, and of the endogenous tissue inhibitor *TIMP-1* and a marked reduction in migration through Matrigel-coated filters59. Furthermore, in a human prostate cancer cell line, S100A4 suppression significantly reduced the expression and proteolytic activity of MMP-9. Prostate cancer cells overexpressing the *S100A4* gene also significantly expressed *MMP-9 and TIMP-1* genes with increased proteolytic activity of MMP-947.

Regardless of the mechanism of action, it is clear from our present results that high expression of *S100A4* is associated with clinical and pathological features linked to poor prognosis. Importantly, we have also demonstrated here by mechanistic studies that *S100A4* is an important effector of endometrial cancer cell invasion. From the clinical perspective, there are currently no drugs that specifically target S100A4. However, there are drugs in development for fibrotic diseases that target TGF-β receptors60; such drugs may potentially be useful for treatment of advanced endometrial cancer. Furthermore, we have demonstrated here that measuring tumor *S100A4* expression has prognostic value. Endometrial cancer patients with high tumor levels of *S100A4* may therefore benefit from post-operative adjuvant chemotherapy.

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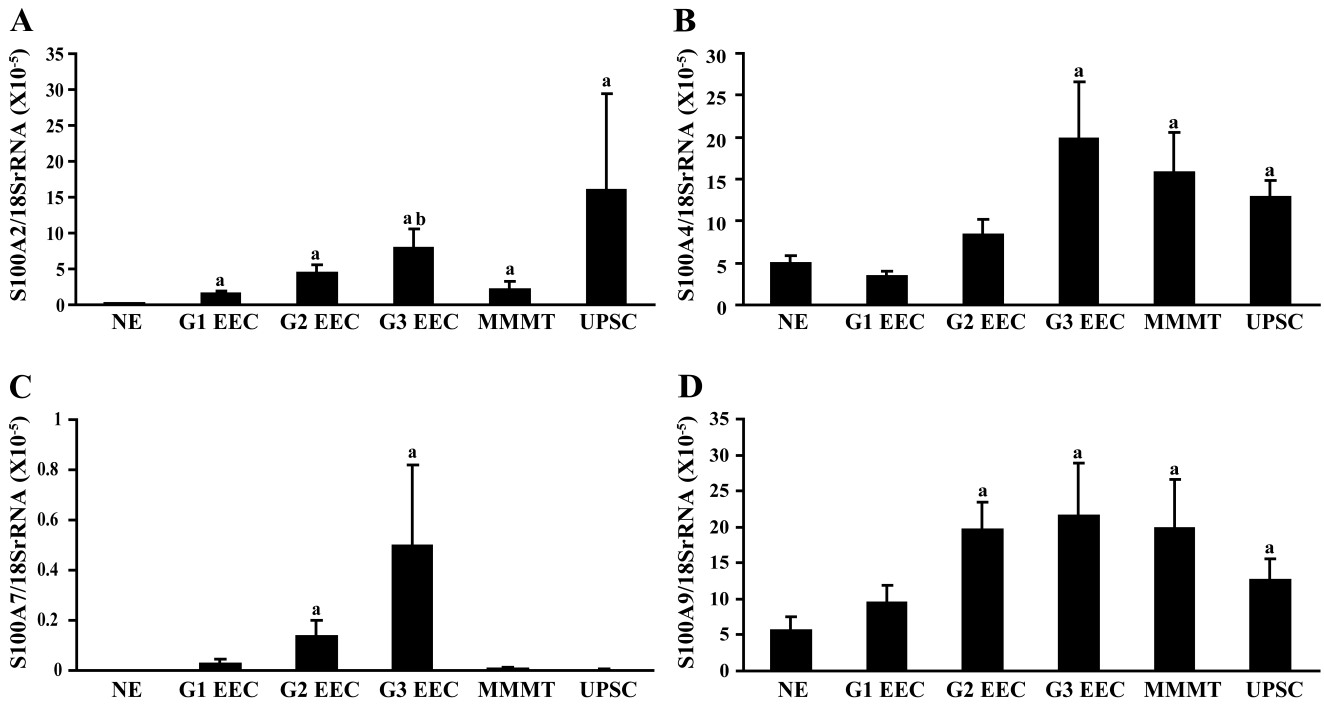

Figure 1.

qRT-PCR analysis of *S100* family members in benign endometrium and endometrial cancer. *S100A* transcripts were measured by qRT-PCR in normal endometrium (NE; n=17), grade 1 endometrioid adenocarcinoma (G1 EEC; n=14), grade 2 endometrioid adenocarcinoma (G2 EEC; n=34), grade 3 endometrioid adenocarcinoma (G3 EEC; n=23), malignant mixed mullerian tumor (MMMT; n=11), and uterine papillary serous carcinoma (UPSC; n=10). Final transcript data were normalized to 18S rRNA and are presented as the molecules of transcript/molecules of 18S rRNA. Values shown are mean ± SE. **A.** *S100A2* was over-expressed in all subtypes of endometrial cancer compared to benign endometrium (a, $p<0.01$). *S100A2* levels in G3 EEC were significantly higher than those for G1 EEC (b, $p=0.007$). **B.** *S100A4* was significantly over-expressed in G3 EEC, MMMT, and UPSC compared to benign endometrium, G1 EEC and G2 EEC (a, $p<0.01$). **C.** *S100A7* was over-expressed in G2 EEC and G3 EEC compared to NE (a, p…0.01), but not in the non-endometrioid tumors UPSC and MMMT. **D.** *S100A9* was significantly increased in G2 EEC, G3 EEC, MMMT, and UPSC compared to NE (a, p…0.05).

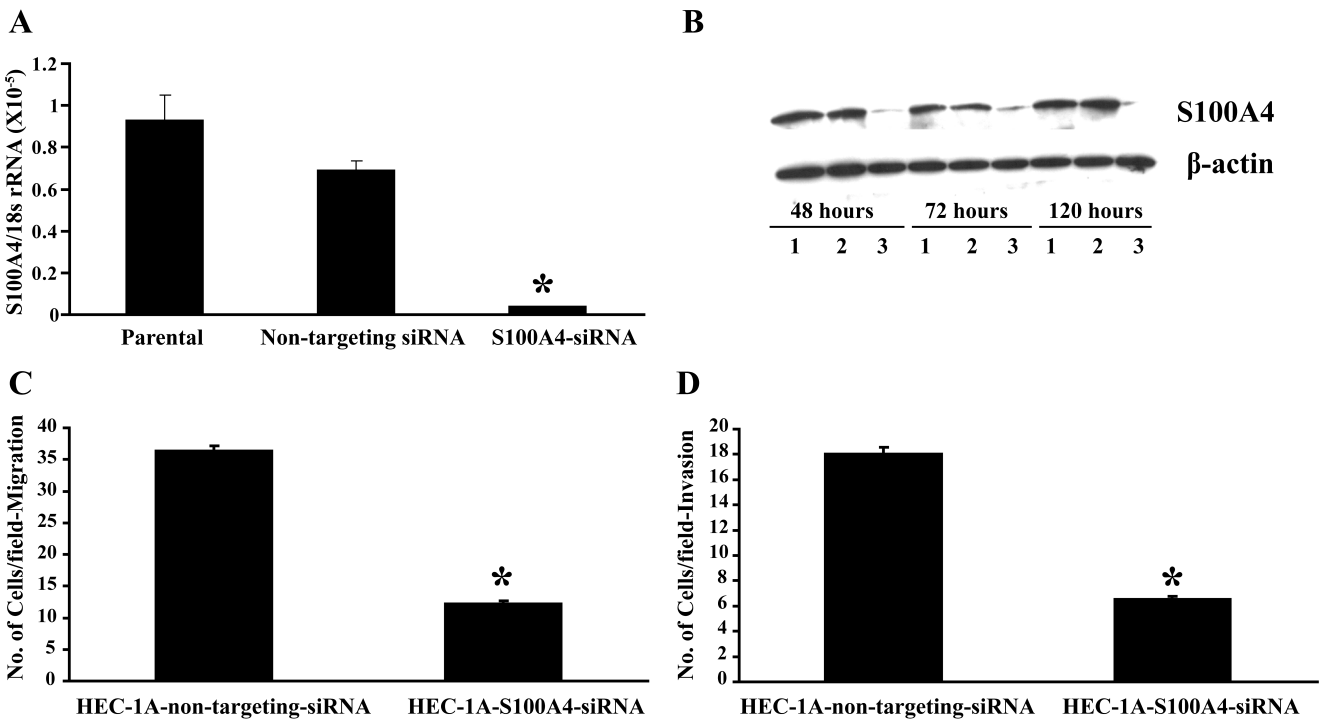

Figure 2.

S100A4 gene knockdown by siRNA transfection in HEC-1A cells. **A.** By qRT-PCR, *S100A4* mRNA expression was significantly suppressed at 36 hours post-transfection of *S100A4* siRNA into HEC-1A cells as compared to non-targeting siRNA transfected cells and parental cells. **B.** By Western blot analysis, S100A4 protein expression was decreased in HEC-1A cells at 48 hours post-transfection of *S100A4* siRNA and remained at low levels at 120 hours post-transfection. Lane 1, HEC-1A parental cells; Lane 2, Non-targeting siRNA transfected HEC-1A cells; Lane 3, *S100A4* siRNA transfected HEC-1A cells. For qRT-PCR, data were normalized to 18S rRNA and are presented as the molecules of transcript/ molecules of 18S rRNA. Values shown are mean ±SE. For Western blot analysis, equal loading of protein was confirmed by stripping the blots and reprobing with β-actin antibody. **C and D.** Knockdown of S100A4 protein in HEC-1A endometrial cancer cells resulted in decreased migration (C) and invasion (D). HEC-1A endometrial cancer cells were transfected with control non-targeting siRNA or *S100A4* siRNA at 48 hours post-transfection. Following microscopic examination, migratory and invasive potentials of the cells were quantified. The invasion and migration assays were performed in triplicate, and means were used in statistical analysis (*, $p<0.001$).

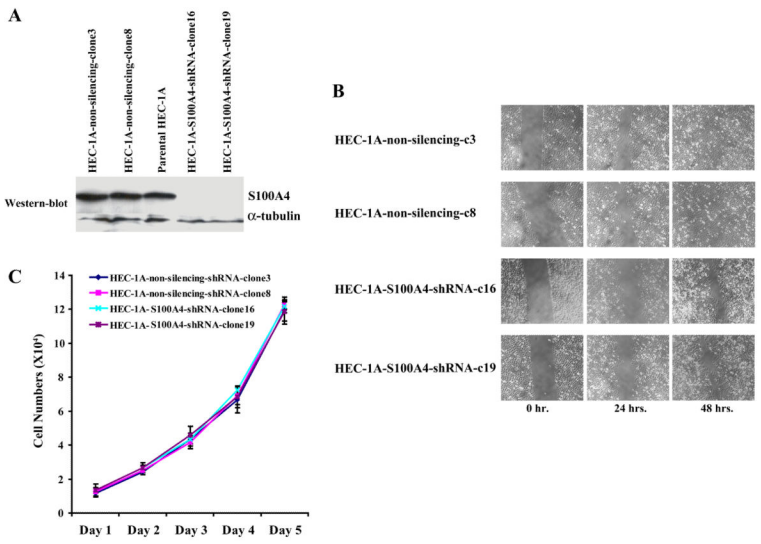

Figure 3.

Endometrial cancer cell migration and proliferation following *S100A4* gene knockdown by shRNA transfection. **A.** By Western blot analysis, S100A4 protein expression was undetectable in two HEC-1A clones (c16 and c19) stably transfected with shRNA for *S100A4* as compared to empty vector controls (c3 and c8) and parental cells. Equal loading of protein was confirmed by stripping the blots and reprobing with α-tubulin. **B.** Wound-healing assay with *S100A4* shRNA stable clones (HEC-1A-*S100A4*shRNA c16 & HEC-1A-*S100A4*shRNA c19) and empty vector clones (HEC-1A-non-silencing shRNA c3 & HEC-1A-non-silencing shRNA c8). A scratch was introduced in the middle of confluently grown cells. Photomicrographs were taken at designated time points to assess the ability of different cell types to heal the wound. **C.** Knockdown of S100A4 had no effect on cell proliferation *in vitro*. Cell proliferation of *S100A4* shRNA stable clones (HEC-1A-S100A4shRNA clone16 & HEC-1A-S100A4shRNA clone19) and empty vector clones (HEC-1A-non-silencing shRNA clone 3 & HEC-1A-non-silencing shRNA clone 8) was assessed by direct cell counting. Cells were seeded at a density of 10,000 cells in a 12-well plate and counted every day for 5 days. Values are expressed as mean ± SE of triplicate determinations.

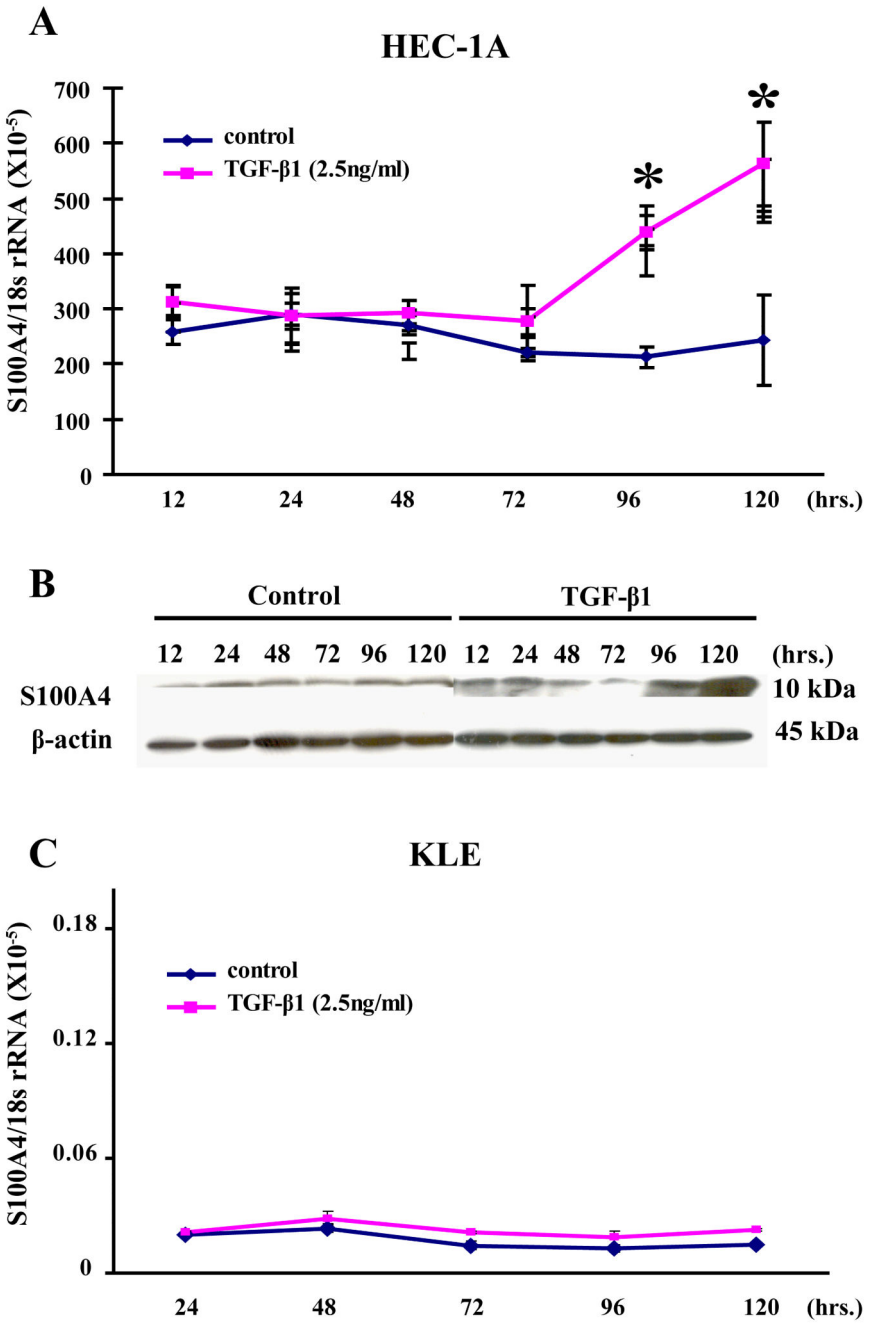

Figure 4.

Up-regulation of *S100A4* expression following TGF-β1 treatment. Cells were treated with TGF-β1 (2.5 ng/ml) or vehicle (4 mM HCl with 1 mg/mL bovine serum albumin) in the absence of serum for the designated time intervals**. A.** *S100A4* mRNA level was induced 72 hours after TGF-β1 treatment and remained elevated at 120 hours post-treatment (qRT-PCR; *, $p<0.05$). **B.** In HEC-1A cells, S100A4 protein was up-regulated following 96 hours of TGF-β1 treatment. β-actin was used as a loading control. **C.** TGF-β1 treatment did not

induce *S100A4* in KLE cells, which we have previously shown to have methylation of *S100A4*6.

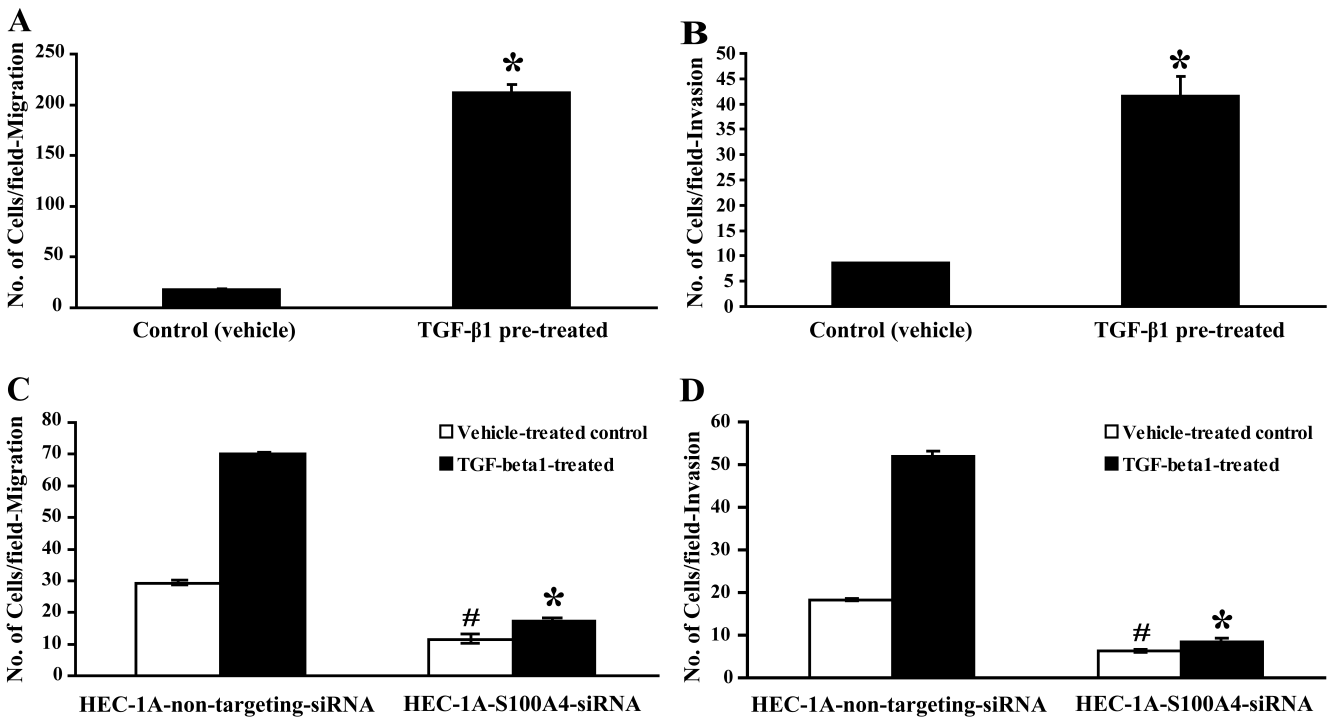

Figure 5.

Chronic TGF-β1 treatment stimulates endometrial cancer cell migration and invasion. **A. and B.** HEC-1A cells were pre-treated with 2.5 ng/ml of human TGF-β1 or an equal volume of TGF-β1 vehicle (4 mM HCl with 1 mg/mL bovine serum albumin) for 5 days. The cells were harvested by brief treatment with trypsin-EDTA and equal numbers of treated or untreated cells were subjected to migration (**A**) or invasion (**B**) assays. Quantification was performed by counting the stained cells using light microscopy. The assays were performed in triplicate, and means were used in statistical analysis. (*, $p<0.05$). **C and D.** Knockdown of *S100A4* inhibits TGF-β1 stimulated endometrial cancer cell migration and invasion. At 36 hours post-transfection of non-targeting siRNA or *S100A4* siRNA, cells were pre-treated with TGF-β1 or an equal volume of TGF-β1 vehicle for 5 days. Migration (**C**) and invasion (**D**) assays were performed as described above. (*, $p<0.05$, TGF-β1 treated HEC-1A-*S100A4*-siRNA vs. TGF-β1 treated HEC-1A-non-targeting-siRNA control; #, $p<0.05$, vehicle treated HEC-1A-*S100A4*-siRNA vs. vehicle treated HEC-1A-non-targeting-siRNA control).

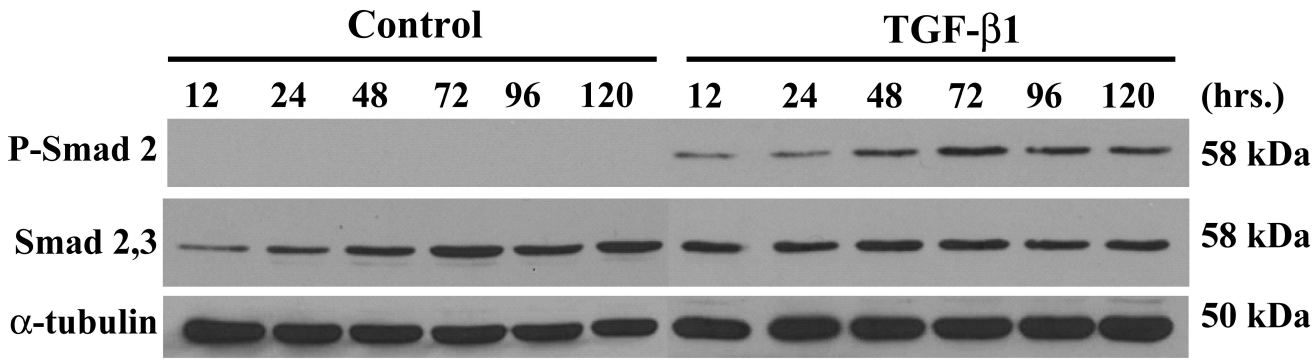

Figure 6.

TGF-β1 induces phosphorylated Smad2 in HEC-1A cells. HEC-1A cells were treated with TGF-β1 (2.5 ng/ml) or vehicle (4 mM HCl with 1 mg/mL bovine serum albumin) in the absence of serum for the indicated time periods. Western blot revealed the presence of phosphorylated Smad2 following TGF-β1 stimulation, whereas the level of total Smad2/3 was similar between vehicle control and TGF-β1 treated HEC-1A cells. No significant differences in total AKT, pAKT, total Erk1,2, and pERK1,2 were observed in TGF-β1 treated cells (data not shown).

Table 1

Probes and primers for real-time quantitative RT-PCR assays

Transcript	Taqman primers and probe	Accession Number
18S rRNA	(535+) GAGGGAGCCTGAGAAACGG	M10098
	(602-) GTCGGGAGTGGGTAATTTGC	
	(555+) FAM-TACCACATCCAAGGAAGGCAGCAGG-BHQ1	
	Lowest quantifiable level = 210 molecules; Average assay efficiency = 104%	
S100A4	69(+) CATGGCGTGCCCTCTG	NM_002961
	133(-) TGCCCGAGTACTTGTGGAAG	
	89(+) FAM-AGGCCCTGGATGTGATGGTGTCC-BHQ1	
	Lowest quantifiable level = 210 molecules; Average assay efficiency=98%	
S100A6	107(+) CATGCCCCCTGGATCAG	NM_014624
	166(-) TGCCGGAGTACTTGTGGAAG	
	126(+) FAM CATTGGCCTCCTCGTGGCCA BHQ1	
S100A7	3(+) CCAAACACACACATCTCACTC	NM_002963
	68(-) TGCTTTCAAAAAGCCAGAG	
	26(+) FAM CCTTCTACTCGTGACGCTTCCCA BHQ1	
	Lowest quantifiable level = 221 molecul; Average assay efficiency =96%	
S100A9	271(+) TTCGAGGAGTTCATCATGCTG	NM_002965
	333(-)CTCGTGCATCTTCTCGTGG	
	293(+)FAM TGGCGAGGCTAACCTGGGCC BHQ1	
	Lowest quantifiable level= 228 molecules; Average assay efficiency=94%	
S100A10	278(+) TGAAGGACCTGGACCAGTGTAG	NM_002966
	350(-) GTGAGGCCCGCAATTAGG	
	302(+)FAM ATGGCAAAGTGGGCTTCCAGAGCTT BHQ1	
	Lowest quantifiable level= 200 molecules ; Average assay efficiency =96%	
S100A11	101(+) GCGCCTCGCTCAGCTC	NM_005620
	164(-)TCGATGCACCGCTCAGTC	
	118(+)FAM AACATGGCAAAAATCTCCAGCCCTACAGA BHQ1	
Snail	942(+) CATCCTTCTCACTGCCATG	NM_005985
	1021(-)GTCTTCATCAAAGTCCTGTGG	
	963(+) FAM-ATTCCCTCCTGAGTGCCCCA-BHQ1	
	Lowest quantifiable level=180 molecules; Average assay efficiency=93%	

Table 2

qRT-PCR expression of S100 gene family members in association with clinicopathological indicators of endometrial cancer invasiveness[a].

	Deep Myometrial Invasion[b]		Lymphatic/Vascular Space Invasion		Extra-uterine Corpus Metastasis	
	Absent	Present	Absent	Present	Absent	Present
S100A4	3.9 ± 0.7	14.9 ± 4.7*	5.1 ± 1.1	16.5 ± 4.6*	7.0 ± 1.4	19.0 ± 6.0*
S100A2	3.7 ± 0.9	6.1 ± 2.4	1.5 ± 0.3	7.0 ± 2.2*	2.9 ± 0.6	8.4 ± 3.0*
S100A7	0.1 ± 0.06	0.1 ± 0.03	0.01 ± 0.01	0.1 ± 0.05*	0.06 ± 0.03	0.1 ± 0.05*
S100A9	16.1 ± 4.4	17.1 ± 3.1	9.8 ± 2.0	23.6 ± 5.6*	12.7 ± 2.7	28.8 ± 6.9*

[a] Final transcript data were normalized to 18S rRNA and presented as [(molecules of transcript/molecules of 18S rRNA) × 10^{-5}]. Values shown are mean ± SE.

[b] Deep myometrial invasion is defined as invasion greater than or equal to 50% myometrial wall thickness.

* $p<0.05$