Functional Interaction Between S100A1 and MDM2 May Modulate p53 Signaling in Normal and Malignant Endometrial Cells

Mayu Nakagawa

Kitasato University School of Medicine

Shyoma Higuchi

Kitasato University School of Medicine

Miki Hashimura

Kitasato University School of Medicine

Yasuko Oguri

Kitasato University School of Medicine

Toshihide Matsumoto

Kitasato University School of Allied Health Science

Ako Yokoi

Kitasato University School of Medicine

Yu Ishibashi

Kitasato University School of Medicine

Takashi Ito

Kitasato University School of Medicine

Makoto Saegusa (✉ msaegusa@med.kitasato-u.ac.jp)

Kitasato University School of Medicine

Research Article

Keywords: S100A1, MDM2, p53, menstrual cycle, endometrial carcinoma

Posted Date: November 10th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1026301/v1>

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Version of Record: A version of this preprint was published at BMC Cancer on February 18th, 2022. See the published version at <https://doi.org/10.1186/s12885-022-09249-1>.

Abstract

Background

S100A1 expression is deregulated in a variety of human malignancies, but its role in normal and malignant endometrial cells is unclear.

Methods

We used endometrial carcinoma (Em Ca) cell lines to evaluate the physical and functional interaction of S100A1 with p53 and its negative regulator, mouse double minute 2 (MDM2). We also evaluated the expression of S100A1, p53, and MDM2 in clinical samples consisting of 89 normal endometrial and 189 Em Ca tissues.

Results

S100A1 interacted with MDM2 but not p53 in Em Ca cell lines. Treatment of cells stably overexpressing S100A1 with Nutlin-3A, an inhibitor of the p53/MDM2 interaction, increased expression of p53-target genes including *p21^{waf1}* and *BAX*. S100A1 overexpression enhanced cellular migration, but also sensitized cells to the antiproliferative and proapoptotic effects of Adriamycin, a genotoxic agent; these phenotypes were abrogated when S100A1 was knocked down using shRNA. In clinical samples from normal endometrium, S100A1 expression was significantly higher in endometrial glandular cells of the middle/late secretory and menstrual stages when compared to cells in the proliferative phases; high S100A1 was also positively correlated with expression of MDM2 and *p21^{waf1}* and apoptotic status, and inversely correlated with Ki-67 scores. However, such correlations were absent in Em Ca tissues.

Conclusion

The interaction between S100A1 and MDM2 may modulate proliferation, susceptibility to apoptosis, and migration through alterations in p53 signaling in normal-but not malignant-endometrial cells.

Background

Endometrial carcinoma (Em Ca) is the most prevalent malignancy of the female genital tract in developing countries [1, 2]. In Japan, the age-adjusted prevalence of Em Ca for women in 2014 was 16.0 per 100,000. The overall rate has increased four-fold in the past 30 years, with a particularly rapid increase in women under 40 years old [3, 4]. Although most Em Ca patients are diagnosed at an early stage, 15-20% of these tumors are advanced or recurrent diseases and are associated with a 5-year

survival rate of 17% [5]. Therefore, there is an urgent need to identify novel biomarkers or therapeutic targets for diagnosis or treatment.

The S100 protein family is comprised of multigene calcium-binding proteins of the EF-hand type and has more than 25 members, each encoded by separate gene clusters on chromosome 1q21. This region is prone to chromosomal rearrangements and is frequently rearranged in cancers [6, 7]. Although S100 family members are highly identical at the sequence and structural level, they are not functionally interchangeable [8–10]. The proteins modulate a plethora of biological processes including proliferation, migration and invasion, inflammation, and differentiation. These effects are mediated via the interaction of S100 protein with a variety of protein targets including the tumor suppressor, p53, and its negative regulator, mouse double minute 2 (MDM2) [11].

S100 expression is deregulated in several human malignancies including carcinomas of the head, neck, lung, and breast [8–10]. However, the precise pattern of S100 expression and biological activity varies in a stage and subtype-specific manner [8–10]. In gynecologic malignancies, analysis of S100 family members may facilitate diagnosis of ovarian carcinoma and help to refine prognosis [12]. However, a greater understanding of their functional roles in normal and malignant endometrium is still required.

Based on our previous works [13, 14], we hypothesized that S100 family members might regulate endometrial glandular cell biology and tumorigenesis through modulation of the p53/MDM2 axis. To test this, we first examined the expression of several S100 family members in Em Ca cell lines, and interrogated the TCGA data to determine whether S100 expression had prognostic significance in Em Ca. We subsequently focused on an association between S100A1 and p53/MDM2, and determined its functional impact on proliferation, apoptosis, and migration in normal and malignant endometrial cells.

Methods

Plasmids and cell lines

FLAG-tagged S100A1 expression plasmid generated by polymerase chain reaction (PCR) was cloned into p3xFLAG-CMV14 vector (Sigma-Aldrich Chemicals, St. Louis, MO, USA). S100A1-specific short hairpin RNA (shRNA) oligonucleotides were designed as described previously [14]. Single-stranded S100A1 oligonucleotides were annealed and then cloned into *Bam*H1-*Eco*RI sites of RNAi-Ready pSIREN-RetroQ vector (Takara, Shiga, Japan), according to the manufacturer's instructions. The primer sequences for the PCR reaction used in this study are listed in Table 1.

Eight Em Ca cell lines (Hec1B, Hec6, Hec108, Hec116, Hec155, Hec251, Hec265 and Ishikawa) were used as described previously [15]. S100A1 expression plasmid or empty vector was transfected into Hec6 and Ishikawa cells (which lack endogenous S100A1 expression) and stable overexpressing clones were established. Conversely, we using an shRNA targeting the *S100A1* gene [13, 16] to reduce the levels of S100A1 in Hec251 cells, which have relatively high endogenous S100A1 expression (Additional file 3: Figure S3A). These cells are referred to as H251-S100A1-knockdown (KD) in the manuscript.

Antibodies and reagents

Anti-FLAG M2 and anti- β -actin antibodies were purchased from Sigma-Aldrich Chemicals. Anti-p21^{waf1}, anti-cyclin D1, anti-p53, anti-BCL2, and anti-Ki-67 antibodies were obtained from Dako (Copenhagen, Denmark). Anti-p27^{kip1} and anti-BAX antibodies were from BD Biosciences (San Jose, CA, USA). Anti-MDM2 and anti-S100A1 were from Abcam (Cambridge, MA, USA). Anti-cleaved caspase-3 and anti-cleaved poly (ADP-ribose) polymerase 1 (PARP1)(Asp214)(D64E10) were from Cell Signaling (Danvers, MA, USA). Anti-cyclin B1 and anti-cyclin A2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Novocastra (Newcastle, UK), respectively.

Rapamycin, aphidicolin, and nocodazole for synchronization of cells at G1, early S, and G2/M phases, respectively, were obtained from Calbiochem (Cambridge, MA, USA). Adriamycin (ADR) and Nutlin-3A were from Sigma-Aldrich Chemicals.

Reverse transcription (RT)-PCR

cDNA was synthesized from 2 μ g of total RNA. Amplification by RT-PCR was carried out in the exponential phase to allow comparison among cDNA synthesized from identical reactions using specific primers (Table 1). Primers for the *GAPDH* gene were also used as described previously [13, 16].

Western blot assay and immunoprecipitation

Total cellular proteins were isolated using RIPA buffer [20 mM Tris-HCl (pH 7.2), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate]. Cytoplasmic and nuclear fractions were prepared using ProteoExtract Subcellular Proteome Extraction kit (Merck KGaA, Darmstadt, Germany). Proteins were resolved by SDS-PAGE, transferred to membranes, and probed with primary antibodies coupled to the ECL detection system (Amersham Pharmacia Biotechnology, Tokyo, Japan).

For immunoprecipitation, cells overexpressing FLAG-tagged S100A1 were lysed with IP buffer [10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 10% NP-40] in the presence of 1 mM CaCl_2 . Cell lysates were cleared and incubated with anti-FLAG M2, anti-p53 or anti-MDM2 antibodies, followed by incubation with Protein G-Sepharose (Amersham Pharmacia Biotechnology). Western blot assay was subsequently performed with anti-FLAG M2, anti-p53, and anti-MDM2 antibodies.

Flow cytometry

Cells were fixed using 70% alcohol and stained with propidium iodide (Sigma) for cell cycle analysis. Cells were analyzed by flow cytometry using BD FACS Calibur (BD Biosciences) and CellQuest Pro software version 3.3 (BD Biosciences).

Apoptotic index

Apoptotic cells were identified in hematoxylin-eosin (HE)-stained sections according to the criteria of Kerr et al. [17]. Five fields were randomly selected, and the number of apoptotic cells was calculated by counting the mean number of apoptotic cells per high power field (HPF).

Immunofluorescence

After transfection of FLAG-tagged S100A1, the cells were incubated with anti-FLAG M2 antibody. FITC- or rhodamine-labeled anti-mouse or rabbit IgG secondaries (Molecular Probes, Leiden, The Netherlands) were used as described previously [13].

Wound healing assay

Cells were seeded into 24-well tissue culture plates and grown to reach almost total confluence. After a cell monolayer formed, a scratch wound was made with a sterile 200- μ l tip. The area of the wound was analyzed by ImageJ software version 1.41 (NIH). Cell migration was calculated based on the number of pixels occupied by the wound closure compared to control scratches.

Migration assay

Cell migration was determined using 24-well transwell chambers with 8- μ m pore size (Corning, NY, USA). The lower chamber was filled with medium containing 10% serum. Cells were suspended in the serum-free upper medium and transferred into the upper chamber. After 48 h, the number of HE-stained cells on the bottom surface of the polycarbonate membranes was counted using a light microscope.

Clinical cases

Histological findings were reviewed in hysterectomy specimens of endometrioid-type Em Cas from the case records of Kitasato University Hospital during the period from 2007 to 2020, according to the criteria of the 2014 World Health Organization classification [18]. Each case was also staged according to the 2009 International Federation of Gynecology and Obstetrics (FIGO) staging system and the TNM classification [19]. A total of 189 Em Ca cases, including 109 of grade (G)1, 51 of G2, and 29 of G3 were investigated. The mean age of the patients was 59.1 years (range from 31 to 92), and 113 were post-menopausal. In addition, our cases included 136 subcategorized as clinical FIGO stage I and 40 as stage II-IV, 77 with upper ($<1/2$) myometrial invasion and 98 with lower ($\geq 1/2$) myometrial invasion, as well as 20 that were positive for nodal metastasis and 156 that were negative.

Eighty-nine biopsy specimens of normal endometrial tissues including 32 in the proliferative phase, 41 in the secretory phase (18 early and 23 middle and late), and 16 in the menstrual phase were also investigated. All tissues were routinely fixed in 10% formalin and processed for embedding in paraffin. Approval for this study was given by the Ethics Committee of Kitasato University School of Medicine (B19-144).

Immunohistochemistry (IHC)

IHC was performed using a combination of the microwave oven heating and polymer immunocomplex (Envision, Dako) methods as described previously [15, 16].

For evaluation of IHC findings, scoring of cytoplasmic and/or nuclear immunoreactivity for S100A1, p53, MDM2, p21^{waf1}, and Ki-67 in normal endometrial tissues was performed based on the percentage of immunopositive cells and the immunointensity; the values of these two parameters were multiplied together as described previously [13, 16]. In Em Ca tissues, scoring of S100A1 and p53 immunoreactivities was also carried out in a similar manner. Nuclear immunopositivity for MDM2, p21^{waf1}, and Ki-67 was counted in at least 1000 cells from five randomly selected fields and the labeling indices (LIs) were then calculated as a percentage. In addition, the number of cleaved PARP1-positive cells in five randomly selected fields was used to calculate the mean number of apoptotic figures per HPF.

To examine an association between S100A1 immunoreactivity and several clinicopathological factors, the score was divided into two categories (high and low) with a mean score of 4.5 used as cutoff.

TCGA data analysis

mRNA expression data (RNA Seq V2 PSEM) for the S100 family in 520 cases were extracted from cBioportal for Cancer Genomics (<http://www.cbioportal.org/>). The data were subcategorized into 'high' and 'low' groups (scores >0 and 0, respectively) based on the median Z score (= 0) for mRNA expression levels in each category, and then examined for any correlation with overall survival (OS) or progression-free survival (PFS).

Statistics

Comparative data were analyzed using the Mann-Whitney *U*-test, and Spearman's correlation coefficient. The cutoff for statistical significance was set as $P < 0.05$.

Results

Expression of S100 family members and their prognostic significance in Em Ca

We first examined the expression of 13 S100 family members in eight Em Ca cell lines. S100A10 and S100A11 mRNAs were the most abundant, whereas levels of S100A1, S100A2, S100A4, S100A9, and S100A13 were moderate. There was very little or no expression of S100A3, S100A5, S100A6, S100B, S100CA, and S100P (Additional file 1: Figure S1).

We then analyzed data from TCGA to determine whether any S100 mRNAs had prognostic significance in Em Ca. Among the 18 S100 family members, Kaplan-Meier curve analysis showed that patients with high S100A1, S100A5, and S100P mRNA expression had poorer OS and/or PFS when compared to patients with low expression of these genes (Additional file 2: Figure S2).

Based on the above findings, we therefore focused on the functional role of S100A1 in normal and malignant endometrial cells.

S100A1 interacts with the p53/MDM2 axis in Em Ca cells

Some S100 family members are known to modulate the p53/MDM2 axis [11]. To examine the association between S100A1, p53, and MDM2 in Em Ca cells, we established cell lines stably overexpressing FLAG-tagged S100A1 in Hec6 cells (clones H6-S100A1#55 and #58) and Ishikawa cells (clones Ish-S100A1#14 and #23). Conversely, we also generated two independent Hec251 clones in which S100A1 expression was blocked by an S100A1-specific shRNA (H251-shS100A1#3 and #8) (Additional file 3: Figure S3).

We were able to detect reciprocal pairwise interactions between S100A1, p53, and MDM2 in both stable H6- and Ish-S100A1 cell lines, with the exception of an association between S100A1 and p53 (Figure 1A and Additional file 4: Figure 4A). Treatment of cells with Nutlin-3A, which inhibits the proteasomal degradation of p53 by antagonizing its interaction with MDM2 [20], also increased p53 and MDM2 protein levels. This was accompanied by an increase in the expression of p53 transcriptional targets including p21^{waf1} and/or BAX in S100A1 overexpressing cells as compared to control cells (Figure 1B and Additional file 4: Figure S4B). These findings suggest that S100A1 interacts with MDM2, leading to induction of p53-target molecules in Em Ca cells.

Overexpression of S100A1 is associated with changes in cell proliferation, apoptosis, and migration in Em Ca cells

To examine whether S100A1 modulate proliferation in Em Ca cells, we looked for variations in S100A1 expression during cell cycle progression. Figure 2A shows that the nucleocytoplasmic distribution of S100A1 in cells stably or transiently overexpressing the protein was similar in G1-, early S-, and G2/M-arrested cells; this was consistent with data from immunofluorescence staining (Figure 2B). To further

examine whether S100A1 could alter cell growth and expression of cell cycle-related molecules, we seeded S100A1-overexpressing and S100A1-KD cells at low density and rendered them quiescent by serum starvation before adding back serum to induce cell cycle entry. As shown in Figure 3A (and Additional file 5: Figure S5A), cells stably overexpressing S100A1 had a low proliferative rate, particularly in the exponential growth phase; this was concomitant with increased MDM2 and p21^{waf1} expression. In contrast, H251-S100A1-KD cells proliferated more rapidly, together with decreased p21^{waf1} but not MDM2 expression (Figure 3B).

Next, we examined whether there was an association between S100A1 expression and apoptosis in response to the genotoxin, ADR. Overexpression of S100A1 in Hec6 cells increased the sub-G1 fraction (Figure 4A) and number of apoptotic cells (Figure 4B) following ADR treatment. This was observed together with increased expression of p53, MDM2, BAX, and cleaved caspase-3, and with decreased BCL2 expression (Figure 4C). Similar findings were also observed in ADR-treated Ish-S100A1 cells (Additional file 5: Figure S5B, C, D). In contrast, the sub-G1 fraction and the number of apoptotic cells was reduced when S100A1 levels were reduced with shRNA (Figure 4D, E). Consistent with this, levels of cleaved caspase-3 were lower in ADR-treated H251-S100A1-KD cells (Figure 4F).

Since several S100 proteins can modulate cell mobility [21–23], we examined whether S100A1 specifically regulated cell migration. Overexpression of S100A1 significantly increased the migration rate of cells in a wound assay in both cell types studied (Figure 5A, B and Additional file 6: Figure S6). In contrast, cellular migration capability was significantly decreased when S100A1 was knocked down (Figure 5C).

These findings suggest that S100A1 can modulate proliferation, apoptosis, and migration in Em Ca cells.

IHC findings in normal and malignant endometrial tissue

In the glandular components of normal endometrium, we observed nuclear/cytoplasmic S100A1 immunostaining and nuclear immunoreactivity for p53, MDM2, p21^{waf1}, and Ki-67 (Figure 6A). Average IHC scores for S100A1, MDM2, and p21^{waf1} and apoptotic features were significantly higher in menstrual and/or middle/late secretory phases than in other stages. In contrast, Ki-67 score showed a significant stepwise decrease from proliferative to menstrual phases (Figure 6B). As shown in Table 2, average S100A1 score positively correlated with MDM2 and p21^{waf1} score and with apoptotic features; S100A1 score was inversely correlated with Ki-67 score. p53 score was significantly associated with Ki-67 score and apoptotic status, whereas p21^{waf1} score was significantly associated with MDM2 score and apoptotic cells.

We also examined the coincidence of the above markers with cleaved PARP1, a marker of cells that are undergoing apoptosis [24], in Em Ca tissue. S100A1 and p53 scores, Ki-67 IIs, and cleaved PARP1-positive cells were significantly higher in G3 Em Ca than G1 or G2 tumors, whereas MDM2 score was significantly higher in the latter (Figure 7B). Average S100A1 score was not associated with any of the markers investigated, whereas p53 score positively correlated with Ki-67 IIs and cleaved PARP1-positive

cells (Table 2). There were no associations between S100A1 score and several clinicopathological factors (see Methods and Additional file 7: Table S1).

Discussion

We previously demonstrated that both cytoplasmic and nuclear S100A4 expression is substantially increased in G2/M-arrested Em Ca cells compared with G1- and early S-arrest cells [13]. Since the interaction between S100A1 and S100A4 is mutually antagonistic [25, 26], we expected cyclic S100A1 expression during cell cycle progression. However, our current data reveal that this is not the case. In general, S100A1 is abundantly expressed in cardiomyocytes, skeletal muscle fibers, and neural populations, where it regulates cytoskeletal fibers including microtubules, type III intermediate filaments, and actin [27]. Together with our current results, this therefore suggests that expression of S100A1 (and in particular the cytoplasmic form) may help to establish and maintain endometrial gland cell shape, which in turn promotes a secretory phenotype during normal menstrual cycles.

Consistent with a previous report [28], we found that S100A1 interacts with MDM2, the negative regulator of p53, in Em Ca cells. Moreover, treatment with the MDM2 antagonist, Nutlin-3A [20], selectively increased MDM2 expression in S100A1-overexpressing Em Ca cells. Since this treatment also elicited increased expression of p53 and its target genes including *p21^{waf1}* and *BAX*, we suggest that S100A1 overexpression may increase the cellular fraction of p53-free MDM2. In contrast, we could not demonstrate any interaction between S100A1 and p53 in Em Ca cells, whereas other studies have demonstrated that S100A1 binds to a peptide derived from the C-terminal negative regulatory domain of p53 [29, 30]. This discrepancy suggests that S100A1 may require additional cell- and/or tissue-specific factors to interact with p53.

Interestingly, stable overexpression of S100A1 significantly inhibited cell proliferation, and increased MDM2 and *p21^{waf1}* expression in response to serum stimulation. S100A1 overexpression also sensitized cells to genotoxin-induced apoptosis, probably due to increased BAX and cleaved caspase-3 expression and decreased BCL2 expression. In contrast, S100A1-KD cells elicited opposite effects. From these data, we infer that S100A1 may bind to MDM2 and reduce its ubiquitylation of p53, which in turn leads to reduced proliferation and increased apoptosis, probably due to transcriptional activation of p53-target genes including *p21^{waf1}* and *BAX*. Consistent with this, we found that S100A1 score was significantly associated with MDM2 and *p21^{waf1}* expression, as well as apoptotic status, in normal endometrial tissue during the menstrual cycle.

We also found that S100A1 overexpression enhanced cell migration, whereas S100A1 knockdown reduced motility. These pro-migratory but antiproliferative activities of S100A1 are consistent findings that migratory cells have a lower proliferation rate than those in the less mobile but highly proliferative tumor core [31–33]. Similar findings were also observed in ovarian carcinoma cells, supporting observations that S100A1 plays an important role in tumor cell migration through interaction with different components of the cytoskeleton including microtubule and intermediate filaments [34]. MDM2

induces epithelial-mesenchymal transition by enhancing Snail expression or via activation of Smad2/3 signaling in breast and lung carcinomas [35, 36]. As these phenotypes are also induced by S100A1, and we observe a physical interacting between S100A1, we infer that S100A1 may mediate some of its biological effects via MDM2.

S100A1 expression can have different prognostic significances dependent on the specific type of gynecological malignancy. For example, S100A1 serves as a favorable prognostic factor in endometrioid subtypes of ovarian carcinomas [37], whereas its expression is associated with poor prognosis in ovarian and endometrial endometrioid carcinomas [38]. We found that S100A1 expression was significantly higher in G3 Em Ca as compared to G1 tumors. Moreover, our TCGA data analysis revealed a significant association between S100A1 mRNA expression and poorer OS and PFS in Em Ca. We therefore speculate that high S100A1 expression is an indicator of poor prognosis in Em Ca. However, S100A1 expression level did not correlate with several clinicopathological factors or with the status of MDM2 and other molecules in the p53 pathway in Em Ca tissues. The discrepancies between the results of Em Ca cell line- and tissue-based analyses are probably due to the presence of microenvironmental factors in the latter. For example, interleukin (IL)-6 and IL-8 released from myofibroblasts in the tumor microenvironment induce myeloid cells to differentiate into S100A8/S100A9-expressing myeloid-derived suppressor cells and M2 macrophages [39]. Further studies are now required to more fully elucidate the influence of S100A1 in malignancies, particularly with regard to metastasis and immune surveillance of tumors.

Conclusions

S100A1 expression may modulate cell proliferation, apoptosis and migration through its interaction with MDM2 during the menstrual cycles in normal, but not malignant, endometrial tissue.

Abbreviations

Em Ca: endometrial carcinoma, MDM2: mouse double minute 2, KD: knockdown, cPARP1: cleaved poly (ADP-ribose) polymerase 1, ADR: Adriamycin, HPF: high power field, IHC: immunohistochemistry, OS: overall survival, PFS: progression free survival, IL: interleukin

Declarations

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

MN, SH, and MS carried out the majority of the experiments, analyzed the data, and wrote the manuscript. They were helped by MH, YO, YI, and TI. AY and TM were involved in the study design and data collection. All authors reviewed and approved the final manuscript.

Acknowledgement

We would like to thank Kitasato University Hospital for providing us with equipment and resources.

Funding

This study was supported by a grant from JSPS KAKENHI Grant Number 20K07413. The funding body did not participate in the collection, analysis, interpretation of data or writing of the manuscript. They provided the funds for the authors to conduct the study.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its Additional files.

Ethics approval and consent to participate

Approval for this study was given by the Ethics Committee of the Kitasato University School of Medicine (B19-144). All of our cell lines were commercially purchased and therefore did not require ethics approval for our use.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Tables

Due to technical limitations, tables are only available as a download in the Supplemental Files section.

Figures

Figure 1, Nakagawa

Figure 1

Interactions between S100A1, MDM2, and p53 in Hec6 cells. (A) Western blot analysis (WB) with anti-MDM2 (upper panel), anti-p53 (middle panel), and anti-FLAG M2 antibodies (lower panel) after immunoprecipitation (IP) with the indicated antibodies using stable H6-S100A1#55 cell lysates. Input represents 5% of the total cell extract. Normal rabbit IgG was used as a negative control. In the middle panel (p53), the band indicated by an arrow in the S100A1 lane is non-specific, since the molecular weight is slightly higher than that of endogenous p53 (input). (B) Western blot analysis of the indicated proteins in H6-S100A1 cells in response to Nutlin-3A treatment for the times shown.

Figure 2, Nakagawa

Figure 2

S100A1 expression during cell cycle progression in Hec6 and Ishikawa cells. (A) Stable H6-S100A1#55 (upper) and S100A1-transiently transfected Ishikawa cells (lower) were synchronized in the G1 phase by treatment with 50 nM rapamycin, in the early S phase by 2 μ g/ml aphidicolin, or in the G2/M phase by 0.25 μ g/ml nocodazole for 24 h. Western blot analysis for proteins in the cytoplasmic and the nuclear fractions. (B) After FLAG-tagged S100A1 was transiently transfected into Ishikawa cells, the cells were treated with 50 nM rapamycin, 2 μ g/ml aphidicolin, or 0.25 μ g/ml nocodazole for 24 h and were stained using anti-FLAG M2 antibody. Note the cytoplasmic and nuclear S100A1 staining, independent of the cell cycle phase.

Figure 3, Nakagawa

Figure 3

Changes in proliferation following overexpression and knockdown of S100A1 in Hec6 or Hec251 cells. (A, B) Upper: two independent clones of stable H6-S100A1 (A) and H251-shS100A1 cells (B), respectively, as well as the controls, were seeded at low density. The cell numbers are presented as mean $\pm$ SDs. P0, P3, P6, and P9 are 0, 3, 6, and 9 days after cell passage, respectively. Lower: western blot analysis of the indicated proteins in stable H6-S100A1 cells (A), H251-shS100A1 cells (B), and the controls following re-stimulation of serum-starved (24 h) cells with 10% serum for the indicated times.

Figure 4, Nakagawa

Figure 4

Changes in apoptosis following overexpression and knockdown of S100A1 in Hec6 or Hec251 cells. (A, D) Left: stable H6-S100A1 (A), H251-shS100A1 cells (D), and the controls were treated by 1 μ g/ml Adriamycin (ADR) for the time shown. Daggers indicate sub-G1 fraction. Right: the percentages of cells undergoing apoptosis (sub-G1 fractions) were calculated following flow cytometry. C, control (B, E) Left: after 1 μ g/ml ADR treatment, stable H6-S100A1 (B), H251-shS100A1 cells (E), and the controls undergoing apoptosis are indicated by arrows. Original magnification, x400. Right: the numbers of apoptotic cells are shown as mean $\pm$ SDs. Con, control (C, F) Western blot analysis for the indicated proteins in total lysates from stable H6-S100A1 (C), H251-shS100A1 cells (F), and the controls treated with 1 μ g/ml ADR for the times shown.

Figure 5, Nakagawa

Figure 5

Changes in migration following overexpression and knockdown of S100A1 in Hec6 and Hec251 cells. (A, C) Upper: a scratch was made in the middle of a layer of confluent H6-S100A1 (A) or H251-shS100A1 cells (C), and phase contrast images were taken 72 h or 96 h later. The red lines indicate borderlines between confluent cell layers and wound area. Lower: wound areas were calculated using ImageJ software version 1.41 with those at 0 h set as 1. The experiments were performed in triplicate. Data are expressed as mean $\pm$ SDs. (B) Upper panel: stable H6-S100A1 cells and the control cells were seeded in a 24-well transwell plate and incubated for 48 h in medium without serum. Cells that migrated were stained with hematoxylin-eosin and counted using a light microscope. Lower panel: cell numbers are presented. The experiment was performed in duplicate. Con, control

Figure 6, Nakagawa

Figure 6

Immunohistochemistry (IHC) findings in serial sections of normal endometrium. (A) Staining with hematoxylin-eosin (HE) and IHC for the indicated proteins in proliferative, early and late secretory (Sec), and menstrual (Mens) stages. Closed boxes are magnified in the insets. In the HE panels, apoptotic cells are shown in the insets. Original magnification, x200 and x400 (inset). (B) IHC score for the indicated molecules and number of apoptotic cells in normal endometrial glandular cells during the menstrual cycle. Data are expressed as mean $\pm$ SDs. Mid/late Sec, middle and late secretory endometrium

Figure 7, Nakagawa

Figure 7

Immunohistochemistry (IHC) findings in serial sections of Em Ca tissues. (A) Staining with hematoxylin-eosin (HE) and IHC for the indicated proteins in G1 (upper) and G3 (lower) Em Cas. Closed boxes are magnified in the insets. Original magnification, x200 and x400 (inset). (B) IHC score and labeling indices (LIs) for the indicated molecules and number of cleaved PARP1-positive cells in Em Ca tissues. Data are expressed as mean $\pm$ SDs.

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