Expression of a BTB/POZ Protein, NAC1, Is Essential for the Proliferation of Normal Cyclic Endometrial Glandular Cells and Is Up-regulated by Estrogen

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Abstract

Purpose: The purpose of this study was to investigate the expression and localization of NAC1, a member of the BTB/POZ gene family in the human cyclic endometrium.

Experimental Design: NAC1 expression in normal cyclic endometrium was assessed by immunohistochemistry, and data on clinical variables were collected by retrospective chart review. To elucidate the molecular mechanisms of NAC1 expression in the normal endometrium endometrial carcinoma cell lines (Ishikawa, HHUA; ER+, PR+) and primary cultured normal endometria were tested in a sex steroid induction assay and a NAC1 knockdown assay using siRNA.

Results: Expression of NAC1 in glandular cells was significantly higher in the early and mid proliferative phases than in the other menstrual phases. Both NAC1 RNA and protein expression were up-regulated by treatment with 10 nmol/L 17β-Estradiol (E2) in Ishikawa, HHUA and primary cultured normal endometrial cells. The estrogen receptor antagonist ICI 182,780 significantly attenuated E2-induced NAC1 expression. NAC1 gene knockdown inhibited cell growth and induced apoptosis in Ishikawa, HHUA, and normal endometria, all of which expressed NAC1. Furthermore, NAC1 siRNA significantly abrogated estrogen-driven cellular proliferation in Ishikawa, HHUA, and primary cultured normal endometrial cells, whereas the control siRNA had no effect on cell growth in any of these cells.

Conclusions: These findings suggest that NAC1 is functionally involved in E2-induced cell growth of the normal endometrial glandular cells. Because NAC1 is thought to have oncogenic potential, the current findings may provide new insight into the mechanism of estrogen induced endometrial carcinogenesis.

Proliferation and differentiation of the human endometrium are controlled by ovarian steroids via their receptors. Estrogen stimulates the proliferation of both glandular and stromal cells, whereas progesterone inhibits their growth. Various aspects of hormone-induced proliferation and differentiation of the endometrium have been investigated. Studies have examined up- and down-regulation of steroid receptors (1, 2) and the role of growth factors or cytokines (3). The sex steroid-induced events are generally believed to occur via estrogen receptors (ER) and progesterone receptors. The molecular pathways downstream of these receptors that eventually promote the transcription of target genes, however, have not been fully elucidated. Steroid receptor cofactors have recently been identified as important molecules intervening between the receptors and target genes. These cofactors are now functionally divided into two subclasses, i.e., coactivators and corepressors. Cofactors are either activators or repressors for other transcription factors and participate in a transcriptional factor cascade of positive and negative regulators. Only a few factors, however, are directly involved in normal endometrial cycles.

The genes of the BTB/POZ family participate in several cellular functions including proliferation, apoptosis, transcription control, and cell morphology maintenance (4). The BTB/POZ proteins share an evolutionally conserved BTB/POZ protein-protein interaction motif at the NH2 terminal that mediates either homodimer or heterodimer formation (4–6). Some BTB/POZ proteins mediate transcriptional repression through their ability to recruit corepressors (7–10). By serial analysis of gene expression (SAGE) levels of all 130 deduced human BTB/POZ genes, we identified NAC1 as a carcinoma-associated BTB/POZ gene (11). NAC1 is a transcription repressor that is involved in self renewal and the maintenance of pluripotency in embryonic stem cells (12). NAC1 is significantly overexpressed in several types of human carcinomas (11), where it plays a critical role in maintaining tumor cell proliferation and survival (11, 13, 14).
NAC1 expression has not yet been described in the normal human endometrium. The purpose of this study was to investigate the expression and localization of NAC1 in the human endometrium at different phases of the menstrual cycle.

**Materials and Methods**

**Tissue samples and immunohistochemistry.** Fifty-four paraffin-embedded endometrial tissues were obtained from the Department of Obstetrics and Gynecology at Shimane University Hospital. All normal endometrial tissues were taken from patients who had undergone hysterectomy for non-endometrial gynecologic disorders (i.e., uterine leiomyoma, early cervical cancer, and ovarian cancer). None of the study participants had taken exogenous hormones before surgery. All patients had regular menstrual cycles. Diagnosis was based on conventional morphologic examination of H&E-stained sections. All samples were classified according to the criteria described by Noyes et al. (15). Endometrial samples meeting the inclusion criteria were classified into five groups: menstrual (n = 4), early proliferative (n = 10), mid-proliferative (n = 5), late proliferative (n = 10), early-mid secretory (n = 11), and late secretory (n = 14). Acquisition of tissue specimens and clinical information was approved by an institutional review board (Shimane University). The paraffin tissues were organized into tissue microarrays, which were made by removing 3-mm diameter cores of tumor from each block.

The NAC1 mouse monoclonal antibody was a kind gift from Dr. Ie-Ming Shih (Johns Hopkins Medical Institutions, Baltimore, MD). Immunohistochemistry was done on deparaffinized sections using the NAC1 antibody at a dilution of 1:100 and an EnVision+ System peroxidase kit (DAKO). Other antibodies used in this study included SMRT antibody (Affinity Bioreagents; 1:100) and NCoR2 (abCAM; 1:100). After antigen retrieval in a sodium citrate buffer, slides were incubated overnight at 4°C with each antibody. The slides for all samples were evaluated with a light microscope by two researchers; the researchers were blind to the menstrual cycle phase. The antibody staining intensity was then analyzed in the glands and stroma using the HSCORE (16).

$$HSCORE = \sum P_i (i)$$

where \(i\) is the intensity of staining (0, undetectable; 1, weakly positive; 2, moderately positive; 3, intensely positive) and \(P_i\) is a score that is based on the percentage of stained cells for each intensity, varying from 0% to 100%.

**Fig. 1.** Immunoreactivity of NAC1 in normal endometrial tissues. Intense immunoreactivity is present in the nuclei of normal endometrial glandular cells. **A**, a case with strong staining of NAC1 in the early proliferative phase (top left). NAC1 immunointensity is moderate in the late proliferative (top right) and early secretory phases (bottom left). NAC1 immunostaining is undetectable in the late secretory phase (bottom right). **B**, distribution pattern of NAC1 expression across the menstrual cycle.
**Cell culture and treatment.** Ishikawa 3-H-12 cells were provided by Dr. Masato Nishida (Kasumigaura Medical Center, Ibaragi, Japan). HHUA cells were also obtained from Riken Bioresource Center (Ibaragi, Japan). A set of primary cultures was established from normal endometrium including EM-1, EM-2, EM-3, EM-4, EM-5, EM-6, and EM-7. The acquisition of anonyoustissuespecimenswasapprovedby the institutional review board (Shimane University). All of the cells were maintained in DMEM (Life Technologies) supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 µg/mL of streptomycin. Freshly isolated endometrial cells were allowed to grow in culture and were used for experiments within two passages. After two passages, to confirm that the cultures retain original epithelial gland cells, we used immunoseorted system. EM1–7 cells were immuno-sorted using an Ep-CAM antibody bound to Dynal beads (Dynal) following the vendor’s instructions (17). And we confirmed that the cultures retain epithelial cells in the microscope. For estrogen or progesterone induction assays, cells were cultured in phenol red–free medium containing 10% dextran-coated, charcoal-treated, FCS for 48 h, and then incubated with 17β-estradiol (E2) or progesterone (P4). E2 and P4 were purchased from Sigma.

**Western blot analysis.** Cell lysates were prepared by dissolving cell pellets in Laemmli sample buffer (Bio-Rad) supplemented with 5% β-mercaptoethanol (Sigma). Western blot analysis was done on normal endometrial cultures. Similar amounts of total protein from each lysate were loaded and separated on 10% Tris-Glycine-SDS polyacrylamide gels (Novex) and electroblotted to Millipore Immobilon-P polyvinylidene difluoride membrane (Bio-Rad). The membranes were probed with the NAC1 antibody (1:100) followed by a peroxidase conjugated anti-mouse or anti-rabbit immunoglobulin (1:10,000). The same membrane was probed with an antibody that reacted with glyceraldehyde-3-phosphate dehydrogenase for loading controls. Western blots were developed by chemiluminescence (Pierce).

**Quantitative PCR analysis.** Total cellular RNA was isolated using an RNA extraction kit (Qiagen). The samples from the E2 and P4 induction assays were analyzed for NAC1 transcript expression by quantitative real-time PCR using an iCycler (Bio-Rad) with SYBR Green dye (Molecular Probes). Averages in the threshold cycle number of duplicate measurements were obtained. The results were expressed as the difference between the threshold cycle number of the gene of interest and the threshold cycle number of a control gene (APP) for which expression was relatively constant among the serial analysis of gene expression libraries analyzed (18).

**siRNA knockdown of NAC1 gene expression.** Two small interfering RNA sequences (siRNAs) that targeted NAC1 were designed, with sense sequences of UGAUGUACACGUUGGUGCCUGUCACCA and GAGGAAGAAGAACUCGGUCUCUCCAU. Control siRNA (luciferase siRNA) was purchased from IDT. Cells were seeded into 96-well plates and transfected with siRNAs using oligofectamine (Invitrogen). Cell number was determined indirectly by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 72 h after transfection of siRNA (19). Data were expressed as the mean ± 1 SD from triplicates.

**Apoptotic cells were detected using Annexin V and 4,6-diamidino-2-phenylindole staining.** Data were expressed as the mean ± 1 SD from triplicates.

**Immunofluorescence staining.** Cultured cells were fixed with 3% paraformaldehyde for 5 min and were incubated with primary antibodies at the following dilutions: NAC1 at 1:100, SMRT at 1:100, and NcoR2 at 1:100 for 2 h at room temperature followed by incubation with fluorescence-labeled secondary antibodies for 1 h at room temperature. For double staining, cells grown on chamber slides.
were first incubated with NAC1 antibody followed by a Texas red-labeled anti-mouse immunoglobulin and antibodies to SMRT or NcOR2 followed by a FITC labeled anti-rabbit immunoglobulin. Cells were counterstained with 4',6-diamidino-2-phenylindole and the immunofluorescence detected using a Nikon Eclipse 50i fluorescence microscope with appropriate filters.

**Immunoprecipitation.** Coimmunoprecipitation was done to assess whether the corepressors SMRT or NcOR2 were bound to NAC1 by immunoprecipitating cell lysates with a NAC1 antibody followed by immunoblotting with SMRT or NcOR2 antibodies. As a reciprocal assay, we immunoprecipitated the same cell lysates using an SMRT or NcOR2 antibody followed by immunoblotting with the NAC1 antibody.

**Statistical analysis.** Results are expressed as the mean ± SE. A Student’s t test (for comparison of two groups) or one-way ANOVA (for comparison of more than two groups) was used to evaluate numerical data. The $\chi^2$ test or Fischer’s exact test was used for comparisons of categorical data. The correlation coefficient ($r$) between different variables was determined by simple regression analysis. A $P$ value of <0.05 was considered statistically significant.

**Results**

**Expression of NAC1 in normal cyclic endometria.** Immunohistochemistry for NAC1 of the normal endometrium sections revealed strong staining of the glands but almost no staining of the stromal cells in both the proliferative and secretory phases (Fig. 1A). NAC1 protein was detected in the gland cell nuclei. Expression of NAC1 in the gland cells was significantly higher in the early and mid proliferative phases.

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**Fig. 3.** Effects of NAC1 knockdown on cell proliferation and apoptosis in endometrial cancer cell lines and normal cultured endometrial cells. **A,** Western blot analysis showing a significant reduction of NAC1 protein in NAC1 siRNA-treated cells compared with control siRNA-treated cells in Ishikawa cells. NAC1 siRNA significantly reduces cell numbers in Ishikawa. **B,** NAC1 siRNA reduces cell numbers significantly in early proliferative phase cells (EM-3), which overexpress NAC1. **Top left,** H&E stainings showing EM-3 cells were confirmed at the early proliferative phase. **Top right,** NAC1 immunostaining.
than in the other menstrual phases (Fig. 1B). The staining intensity of the stromal cells remained unchanged throughout the menstrual cycle (data not shown).

**Estrogen or progesterone induction assays.** To examine the effect of E2 or P4 on NAC1 expression, ER- and progesterone receptor–positive Ishikawa and HHUA endometrial cancer cells, and primary cultured cells established from normal endometria (EM-1) were cultured in the absence or presence of E2 or P4. NAC1 expression was examined using a quantitative PCR and Western blotting. The addition of E2 at 10 nmol/L up-regulated NAC1 gene and protein expression in a time-dependent manner (Supplementary Fig. S1A and B; Fig. 2A). There was no significant change in NAC1 expression after the addition of 10 nmol/L P4 (Supplementary Fig. S1C). To clarify whether NAC1 induction was mediated by ER signaling, HeLa cells that did not express the ER were used for the E2 induction assay. No significant changes in NAC1 expression were found (Supplementary Fig. S1D). To confirm this finding, we used 1 μmol/L ICI 182,780 (a highly selective ER antagonist) in the E2 induction assay in Ishikawa, HHUA, and primary cultured cells established from normal endometria (EM-2). ICI 182,780 attenuated the E2-induced up-regulation of NAC1 in all cell types (HHUA, EM-2; data not shown; Fig. 2B).

**Functional analysis of NAC1 expression in Ishikawa, HHUA, or cultured normal endometrial cells.** NAC1 was essential for cell growth and survival both in cell lines and in the normal endometrium. NAC1 siRNA was applied to the culture medium of Ishikawa and HHUA endometrial cancer cells. siRNA treatment significantly reduced NAC1 protein expression compared with control siRNA treatment (Fig. 3A). NAC1 siRNA reduced cell numbers significantly in both Ishikawa and HHUA cells (HHUA; data not shown; Fig. 3A). This phenomenon was then confirmed in the normal endometrium by NAC1 gene knockdown. NAC1 siRNA was applied to the culture medium of normal endometrial cells, including early proliferative phase cells (EM-3), midproliferative phase cells (EM-4), and early secretory phase cells (EM-5) that overexpressed NAC1, and one late secretory phase that did not express NAC1 (EM-6). EM-3, EM-4, EM-5, and EM-6 cells were confirmed each menstrual cycle phase using H&E staining (Supplementary Fig. S2A, B, and C; Fig. 3B). NAC1 siRNA treatment significantly reduced NAC1 protein expression compared with control siRNA treatment (Supplementary Fig. S2A and B; Fig. 3B). NAC1 siRNA reduced cell numbers significantly in early proliferative (EM-3), midproliferative (EM-4), and early secretory phase cells (EM-5). This reduction in cell number was not seen in the late secretory phase cells that did not express NAC1 (EM-6; P < 0.05, Student’s t test; Supplementary Fig. S2A, B, and C; Fig. 3B). The inhibition of cell growth after repression of NAC1 expression in Ishikawa, HHUA, and early proliferative phase cells (EM-3) likely resulted from the induction of apoptosis. The percentage of apoptotic cells identified using Annexin V staining or 4',6-diamidino-2-phenylindole staining was significantly increased for NAC1 siRNA-treated cells compared with control siRNA-treated cells (Supplementary Fig. S3; Fig. 4).

**NAC1 siRNA attenuates E2-induced cell growth.** Next, we examined whether or not up-regulation of NAC1 by E2 regulated the proliferation of Ishikawa, HHUA, and primary cultured cells established from normal endometria (EM-7). E2 significantly induced cell growth at 10 nmol/L in these cells. We then hypothesized that Nac1 knockdown would reverse cell growth conferred by E2. We treated Ishikawa, HHUA, and EM-7 cells with siRNA targeting NAC1, and as shown in Fig. 5, NAC1

![Fig. 4. Detection of apoptotic cells. A, the experiment was done 72 h after NAC1 siRNA or control siRNA treatment. Many NAC1 siRNA-treated cells (bottom right), but no control siRNA-treated cells, show positive staining for Annexin V (top right). B, treatment with NACT siRNA increases apoptosis of Ishikawa, HHUA, and EM-3 cells as measured by Annexin V staining.](attachment:Fig4.png)
siRNA significantly restored cellular proliferation from E2 induction in all three lines, whereas the control siRNA had no effect on cell growth.

**Relationship between expression of the corepressors SMRT or NcOR2 and NAC1 expression.** Because NAC1 is regarded as a transcriptional repressor (11, 20), we analyzed the relationship between NAC1 expression and the expression patterns of SMRT and NcOR2. The changes in HSCORE for each of the factors in the glandular cells are schematically shown in Fig. 6. No significant associations were found between NAC1 expression patterns and the expression patterns of SMRT and NcOR2 in endometrial gland cells (Fig. 6).

Neither NAC1, SMRT, nor NcOR2 colocalized with one another (Fig. 6). In a coimmunoprecipitation study, NAC1 did not heterodimerize either with SMRT or NcOR2 (data not shown).

**Discussion**

The BTB/POZ gene family members have, in recent years, assumed a more central role in human cancer (21–26). Recently, we reported that NAC1, a gene with oncogenic potential, was associated with gynecologic tumors (11, 14, 20).

However, none of the data to date documents the expression and the role of these proteins in the normal cyclic human endometrium. Additionally, how NAC1 is regulated by ovarian sex steroids is unknown.

The present study describes the expression pattern of NAC1 in different compartments of the human endometrium at various menstrual cycle phases. Using microarray and immunohistochemical staining, we determined that in benign endometrial samples, NAC1 expression in the epithelial cells and stroma was nonsynchronized. It was up-regulated in the endometrial gland epithelium during the proliferative phase to midsecretory phases with the most intensive staining during early proliferative phase. To our knowledge, this is the first report of the fluctuation of NAC1 expression in hormone-targeted tissues.

Because endometrial cells are primarily regulated by ovarian sex steroids, it is likely that hormonally dependent changes across the menstrual cycle modulate NAC1 expression. To elucidate the mechanisms underlying the effects of NAC1 in normal cyclic endometrium, we used two independent but complementary approaches. First, we ectopically added E2 to cultures of Ishikawa, HHUA, and normal endometria. Next, we knocked down NAC1 using siRNA in the above cells. We then...
analyzed the phenotypic changes and NAC1 expression levels in each of these models. Reduction of NAC1 expression resulted in apoptosis in the NAC1-expressing cells, Ishikawa, HHUA, EM-3, EM-4, and EM-5. This indicates that NAC1 is essential for the proliferation and survival of normal endometrial cells.

Up-regulation of NAC1 was only observed after E2 induction but not P4 induction. Because NAC1 expression was up-regulated by E2 in endometrial cancer cells and normal endometria, we tested the hypothesis that NAC1 might promote cell growth in the endometrium in response to E2 stimulation. Expectedly, NAC1 siRNA partially attenuated cellular proliferation after E2 induction in Ishikawa, HHUA, and primary cultured EM-7 cells. Our findings both in vivo and in vitro have important implications concerning hormone-dependent NAC1 expression and help to explain the molecular mechanisms by which NAC1 expression is regulated across the menstrual cycle. NAC1 expression is weak or undetectable during menstruation. However, once the proliferative phase begins, NAC1 is dramatically up-regulated. Expression is maximal in the early proliferative phase with a subsequent reduction in expression in the secretory phase. Interestingly, similar changes in the expression of c-Myc and telomerase are seen in the endometrium in response to E2 (27).

Prolonged exposure to estrogen is one of the risk factors for the development of endometrial cancer, a malignancy that is becoming increasingly common (28). Although the effects of estrogen on the proliferation of endometrial cells are complex, up-regulation of NAC1 by estrogen may contribute to estrogen-induced endometrial carcinogenesis. Further study is needed to clarify the relationship between estrogen-induced endometrial

Fig. 6. Immunoreactivity of SMRT and NcOR2 in normal endometrial tissues. Intense immunoreactivity is present in the nuclei of normal endometrial glandular cells. A, a case with positive staining of SMRT in early proliferative phase cells. B, a case with positive staining of NcOR2 in early proliferative phase cells. C, the changes in HSCORE of each factor in the glandular cells are schematically shown. D, NAC1 immunofluorescence staining (left). Middle, SMRT immunofluorescence staining. Right, NAC1 does not colocalize with SMRT after double immunofluorescence staining in HHUA cells.
carcinogenesis and NAC1 overexpression using endometrial cancer specimens.

Recent research into steroid receptor–related signaling has identified a group of molecules termed steroid receptor cofactors (29–31). These factors bind to steroid receptors in a ligand-dependent fashion. The receptor-bound cofactors then bind to the basal transcriptional machineries of the target genes and facilitate transcription. A key question is how estrogen regulates NAC1. The ER antagonist ICI 182,780 significantly attenuated E2-induced NAC1 expression. Furthermore, HeLa cells lacking the ER failed to show E2-induced NAC1 expression. These results associate NAC1 expression with ER signaling. NAC1 protein has recently been shown to have a function similar to that of the transcriptional repressor BCL-6 (11). BCL-6 is the most well-characterized of the BTB/POZ genes. Its protein mediates transcriptional repression by recruiting silencing mediator for retinoid and thyroid hormone receptor (SMRT)/nuclear receptor corepressor (NcOR) along with mSin3a and the histone deacetylase complex (7–9).

Therefore, we analyzed the relationship among the expression levels of NAC1 and the corepressors SMRT and NcOR2. There was, however, no positive correlation between NAC1 expression and the above corepressor proteins in the normal cyclic endometrium. Furthermore, NAC1, SMRT, and NcOR2 failed to colocalize with one another in an immunoprecipitation study. Although no other proteins coimmunoprecipitated with NAC1, NAC1 may have formed heterodimers with other BTB/POZ domain-containing proteins, albeit with a lower binding affinity than that seen with homodimers (11). Further examination is needed to identify whether NAC1 expression is regulated by signaling through other steroid hormone receptors.

In summary, the present study showed that NAC1 was overexpressed in the normal endometrium in the early and mid proliferative phases. Expression was up-regulated by E2 and involved in E2-induced cell growth in endometrial cells. NAC1 expression was essential for growth and survival in the normal endometrium. Because NAC1 is considered to have oncogenic potential (11, 14, 20), the current findings may provide new insight into understanding the mechanism behind estrogen-induced endometrial carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

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