Nucleobindin 2 (NUCB2) in human endometrial carcinoma: a potent prognostic factor associated with cell proliferation and migration

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Abstract. Nucleobindin 2 (NUCB2) is a multifunctional protein containing several functional domains, and associated with a wide variety of biological processes such as food intake and energy homeostasis. Recently, NUCB2 has been implicated in not only normal human tissues but also some kinds of human malignancies. However, its clinical and/or biological significance has largely remained unknown in endometrial carcinomas. We therefore immunolocalized NUCB2 protein in 87 endometrial carcinoma tissues and examined its clinical significance. NUCB2 immunoreactivity was detected in 19 out of 87 (22%) of endometrial carcinoma cases examined, and positively correlated with Ki67 labeling index, while there was no significant correlation between NUCB2 and stage, histological grade, and progesterone receptor status. Furthermore, NUCB2 immunoreactivity was significantly correlated with an increased risk of recurrence and worse clinical outcome regardless of stage or histological grade. Subsequent multivariate analyses did reveal that NUCB2 immunoreactivity was an independent prognostic factor for both disease-free survival and endometrial cancer specific survival. In vitro experiments demonstrated that knockdown of NUCB2 using specific siRNA for NUCB2 significantly impaired cell proliferation and migration of the endometrial carcinoma cell lines, Ishikawa and Sawano cells, and that nesfatin-1 treatment significantly promoted cell proliferation and migration in Ishikawa cells. These findings possibly suggested that NUCB2 and/or nesfatin-1 had pivotal roles in the progression of endometrial carcinomas. Immunohistochemical NUCB2 status may therefore serve as a potent biomarker for endometrial carcinomas.

Key words: NUCB2, Endometrial cancer, Prognosis, Biomarker, Immunohistochemistry

ENDOMETRIAL carcinoma is the most common malignancy of the female genital tract, and recently its incidence, especially that of endometrioid endometrial adenocarcinoma, the most common histological type, has increased. Although the majority of patients are diagnosed with stages I and II, those diagnosed with advanced stages have a poor prognosis and the 5-year overall survival rate is 80.1% for stage III and 13.3% for stage IV, respectively [1]. In addition, 10-17% of the patients suffer recurrence even though they are diagnosed at early stage, indicating that the therapeutic application for these patients still remains to be determined [2-6]. Therefore, it is very important to establish useful biomarkers to correctly predict the recurrence of the condition in patients with endometrial carcinomas.

Nucleobindin 2 (NUCB2) is known as a precursor protein of nesfatin-1, which was originally identified in hypothalamic nuclei and associated with food intake and energy homeostasis [7, 8]. NUCB2 protein...
consists of several functional domains such as a signal peptide, a Leu/Ile rich region, two Ca\(^{2+}\) binding EF-hand domains separated by an acidic amino acid rich region, and a leucine zipper [7-9]. A number of studies subsequently demonstrated NUCB2 expression in peripheral tissues such as stomach, pancreatic islets, testis, and adipose tissues [10-13], and a wide variety of basic cellular functions of NUCB2 including insulin release, adipocyte differentiation and myocardial performance has been proposed (reviewed in Cao et al. [14]). Recently, possible roles of NUCB2 have been proposed in several human malignancies, which to date remain controversial [15-20]. It has been reported that nesfatin-1 suppresses cell proliferation of epithelial ovarian carcinoma and adrenocortical carcinoma cells [19, 20], while NUCB2 promoted cell proliferation and invasion of breast carcinoma cells, and expression of NUCB2 serves as worse prognostic factor in breast and prostate cancer [17, 18]. However, to the best of our knowledge, NUCB2 has not been studied in endometrial carcinomas. In addition, detailed molecular mechanisms by which NUCB2 regulates tumor progression has yet to be clarified despite the fact that the biological functions of NUCB2 may be mediated by its proteolytic derivatives which serve as soluble factors (i.e. nesfatin-1) and also several functional domains which are considered to function in the cytoplasm. Therefore, we examined NUCB2 in endometrial carcinoma using immunohistochemistry and in vitro studies in order to explore the clinical and biological significance of NUCB2.

**Materials and Methods**

**Patients and tissues**

Eighty-seven specimens of endometrial carcinomas were obtained from Japanese patients who underwent surgical treatment from 1993 to 2003 in the Department of Obstetrics and Gynecology, Tohoku University Hospital, Japan. None of the patients received chemotherapy, irradiation, or hormonal therapy before the surgery. A review of the patient charts revealed that 25 patients received adjuvant chemotherapy, 26 patients adjuvant radiotherapy, and 2 patients both chemotherapy and irradiation following surgery. The clinical outcome of the patients was evaluated by disease-free and endometrial cancer-specific survival in this study, which was calculated from the time of initial surgery to the date of recurrence or death from endometrial cancer, respectively. All the specimens had been fixed in 10% formalin and embedded in paraffin wax. All 87 cases were then pathologically diagnosed as endometrioid endometrial carcinomas.

**Immunohistochemistry**

Rabbit polyclonal antibody for NUCB2 was purchased from Aviva Systems Biology (San Diego, CA, USA). Mouse monoclonal antibodies for estrogen receptor (ER; ER1D5), progesterone receptor (PR; MAB429) and Ki-67 (MIB1) were purchased from Immunotech (Marseille, France), Chemicon (Temecula, CA) and DAKO (Carpinteria, CA), respectively.

A Histofine Kit (Nichirei Biosciences, Tokyo, Japan), which employs the streptavidin-biotin amplification method, was used in this study. Antigen retrieval was performed by heating the slides in autoclave at 120°C for 20 min in citric acid buffer (pH 6.0) for staining with antibody as described above. Dilutions of primary antibodies used in this study were as follows: NUCB2, 1/100; ER, 1/50; PR, 1/50; and Ki-67, 1/50 [21]. The antigen-antibody complex was visualized with 3,3′-diaminobenzidine (DAB) and counterstained with hematoxylin. Human stomach tissue was used as a positive control of NUCB2 immunostaining [17]. As a negative control, normal rabbit IgG was used instead of the primary antibody. We also confirmed specificity of NUCB2 immunostaining by antibody absorption test using NUCB2 blocking peptide (Aviva Systems Biology).

NUCB2 immunoreactivity was detected in the cytoplasm of carcinoma cells and also non-neoplastic glands, and the cases that had more than 10% positive carcinoma cells were considered positive for NUCB2 immunoreactivity, according to a previous report [17]. Immunoreactivity for ER, PR, and Ki-67 was detected in the nucleus, and the immunoreactivity was evaluated in more than 1,000 carcinoma cells for each case, and subsequently the percentage of immunoreactivity, i.e. labeling index (LI), was determined. Cases with LI of more than 10% were considered positive for ER and PR in this study [22].

This study was approved by the Ethics Committee of Tohoku University Graduate School of Medicine.

**Cell lines and chemicals**

Ishikawa and Sawano cells were purchased from HPA Culture Collections (Salisbury, UK) and RIKEN Bio Resource Center (Tsukuba, Japan), respectively.
Ishikawa cells were maintained in Minimum Essential Media (MEM) (Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Biosera, Ringmer, UK) and non-essential amino acids (Life Technologies). Sawano cells were maintained in MEM containing 15% FBS. Recombinant human nesfatin-1 was purchased from Cell Sciences Inc. (Canton, MA).

Small interfering RNA (siRNA) transfection
Stealth RNAi siRNA Duplex Oligoribonucleotides for NUCB2 were obtained from Life Technologies. The sequence of siRNA against NUCB2 was as follows. siNUCB2 #1: 5′-UAUCUUCGACUUUCCCA CAGGGUGA-3′ and siNUCB2 #2: 5′-UUGAUUU AGCAUAUCUAAUCUGGG-3′. Universal RNAi Negative Control Duplexes (Sigma-Aldrich, St. Louis, MO, USA) were also used as the negative control (siNC). These siRNAs were transfected with endometrial carcinoma cells using Lipofectamine 3000 reagent (Life Technologies).

Real time PCR
Total RNA was extracted using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH), and cDNA was synthesized using a QuantiTect reverse transcription kit (Qiagen, Hilden, Germany). Real time PCR was performed using the LightCycler Nano system (Roche Diagnostics, Mannheim, Germany) and Quantifast SYBR Green PCR Kit (Qiagen). The primer sequence for NUCB2 and ribosomal protein L13A (RPL13A) were as follows. NUCB2: forward 5′-AAAGAAGAGCTACAACGTCA-3′ and reverse 5′-GTGGCTCAAACTTCAATTC-3′, and RPL13A: forward 5′-CCTGGAGGAGAAGGAAAGA-3′ and reverse 5′-TTGAGGACCTCTGTGTATTTG TCAA-3′. Relative NUCB2 mRNA level was calculated as the ratio of RPL13A mRNA level.

Immunohistochemistry
Ishikawa and Sawano cells were maintained in Cell Culture Slide (SPL Life Sciences, Gyeonggi, Korea), and fixed in 10% formalin for 15 min at room temperature. Immunostaining for NUCB2 was performed by a method similar to immunohistochemistry except for antibody dilution (1/400).

Cell proliferation assay and migration assay
For cell proliferation assay, Ishikawa and Sawano cells were transfected with siNUCB2 #1, siNUCB2 #2 and siNC in a 96-well plate. Cell proliferation was evaluated using Cell counting kit-8 (Dojindo, Kumamoto, Japan).

Cell migration assay was performed using a 24-well plate and Chemotaxis cell (8 µm pore size; Kurabo, Osaka, Japan) according to a previous report [23]. Ishikawa and Sawano cells were plated at the upper chamber, and the cells on the upper surface of membrane were removed after incubation for 48 hr. The migration ability was evaluated as the average number of cells in five middle power fields (X200) randomly selected on the lower surface of membrane.

Statistical analysis
The association between NUCB2 immunoreactivity and clinicopathological parameters was evaluated using \( \chi^2 \) test. Disease-free and cancer-specific survival curves were generated according to Kaplan-Meier method, and statistical significance was calculated using log-rank test. Uni- and multivariate analyses were evaluated using proportional hazard model (Cox). Dunnett’s test was used in the \textit{in vitro} experiments. \( P \) values less than 0.05 were considered statistically significant.

Results

Immunolocalization of NUCB2 in endometrial carcinoma
NUCB2 immunoreactivity was detected in the
cytoplasm of endometrial carcinoma cells (Fig. 1A). NUCB2 immunoreactivity was also slightly detected in non-neoplastic endometrial glands, while it was almost negligible in stroma (Fig. 1B). NUCB2 immunoreactivity was significantly diminished when NUCB2 antibody was absorbed by blocking peptide (Fig. 1C). In the positive control, NUCB2 immunoreactivity was mainly detected in the epithelium of the fundic glands in the stomach (Fig. 1D, left), whereas no significant immunoreactivity was detected in the same areas of the negative control section (Fig. 1D, right).

The associations between NUCB2 status and clinicopathological parameters in endometrial carcinomas are summarized in Table 1. Of 87 cases of endometrial carcinomas examined in this study, 19 cases (22%) were positive for NUCB2 immunoreactivity. NUCB2 status was significantly correlated with Ki67 LI ($P = 0.011$), while no significant association was detected in other parameters including stage and histological grade.

![Fig. 1 Immunohistochemistry for NUCB2 in human endometrial carcinomas.](image)

A: NUCB2 immunoreactivity was detected in the cytoplasm of carcinoma cells. B: NUCB2 immunoreactivity was slightly detected in non-neoplastic endometrial glands, while it was almost negligible in normal endometrium or stroma. C: NUCB2 immunoreactivity was significantly diminished by antibody-absorption using blocking peptide. D: Positive (left) and negative (right) control sections for NUCB2 immunostaining (gastric mucosa). Bar = 100 μm, respectively.
NUCB2 immunoreactivity and clinical outcome of endometrial cancer patients

As shown in Fig. 2A, NUCB2 status was significantly associated with increased risk of recurrence \((P = 0.0004\) by log-rank test), and multivariate analysis revealed that stage, NUCB2 status, and PR status were confirmed as independent prognostic factors for disease-free survival with relative risks over 1.0 (Table 2). Similar tendency was obtained regardless of the clinical stage \((P = 0.0019\) in stage I, II group (Fig. 2C) and \(P = 0.0006\) in stage III, IV group (data not shown)) and histological grade \((P = 0.0042\) in grade 1, 2 group (Fig. 2E) and \(P = 0.002\) in grade 3 group (data not shown)).

The association between NUCB2 status and cancer-specific survival was summarized in Fig. 2B. A significant association was detected between NUCB2 status and adverse clinical outcome of the patients \((P = 0.0004)\). Furthermore, NUCB2 status as well as PR status turned out to be an independent prognostic factor (Table 3). Similar tendency was obtained regardless of the stage \((P = 0.01\) in stage I, II group (Fig. 2D) and \(P < 0.0001\) in stage III, IV group (data not shown)) and histological grade \((P = 0.0071\) in grade 1, 2 group (Fig. 2F) and \(P = 0.0022\) in grade 3 group (data not shown)).

**Effects of NUCB2 on cell proliferation and migration in endometrial carcinoma cells**

In order to further clarify the biological significance of NUCB2 in endometrial carcinoma cells, we conducted in vitro experiments using specific siRNAs for NUCB2. siRNA transfection successfully decreased NUCB2 mRNA level in both Ishikawa cells (siNUCB2 #1; 16.2% and siNUCB2 #2; 11.3% com-
Fig. 2  Disease-free and cancer-specific survival of 87 endometrial carcinoma patients according to NUCB2 immunoreactivity. A, B: NUCB2 immunoreactivity was significantly associated with an increased risk of recurrence ($P = 0.0004$) (A) and worse prognosis ($P = 0.0004$) (B). C-F: Disease-free survival curves (C, E) and cancer-specific survival (D, F) according to NUCB2 immunoreactivity in stage I, II (C, D) and Grade1, 2 (E, F) group. $P$ values were evaluated by log-rank test. Dashed line: negative for NUCB2 immunoreactivity; and solid line: positive for NUCB2 immunoreactivity.
pared to siNC, Fig. 3A, upper panel) and Sawano cells (siNUCB2 #1; 26% and siNUCB2 #2; 30.7% compared to siNC, Fig. 3B, upper panel). Knockdown of NUCB2 protein was confirmed by immunocytochemistry (Fig. 3A and 3B, lower panel).

As shown in Fig. 3C, cell proliferation was significantly suppressed in Ishikawa cells transfected with siNUCB2 #1 and #2 compared to those transfected with siNC (0.15-fold and P < 0.0001 in siNUCB2 #1, 0.59-fold and P < 0.0001 in siNUCB2 #2, 4 days after transfection). Similar tendency was observed in Sawano cells under the same condition (Fig. 3D). Moreover, the number of migrated cells was significantly lower in both Ishikawa cells and Sawano cells transfected with siNUCB2 (Ishikawa cells; 0.39-fold and P < 0.001 in siNUCB2 #1, 0.63-fold and P < 0.05 in siNUCB2 #2 (Fig. 3E) and Sawano cells; 0.14-fold and P < 0.01 in siNUCB2 #1, 0.18-fold and P < 0.01 in siNUCB2 #2 (Fig. 3F)).

We then examined whether nesfatin-1 was associated with the effects of NUCB2 on cell proliferation and cell migration. Firstly, we treated Ishikawa and Sawano cells with conditioned medium from NUCB2-silenced cells. However, it turned out that conditioned medium had no significant effects on cell proliferation and migration (Supplementary Fig. 1). Next, we employed recombinant nesfatin-1 peptide for cell proliferation assay and cell migration assay. As shown in Fig. 4A, nesfatin-1 significantly promoted cell proliferation of Ishikawa cells in a dose-dependent fashion (1.2-fold and P < 0.001 in nesfatin-1 (100 ng/mL) treated group compared to control group (non-treated group)). On the other hand, proliferation of Sawano cells was not significantly changed following nesfatin-1 treatment (Fig. 4B). Similarly, nesfatin-1 significantly promoted cell migration of Ishikawa cells (1.8-fold and P < 0.01, while that of Sawano cells was not (Fig. 4C).

Nesfatin-1 has been reported to be linked to mamma-
Fig. 3 Effects of NUCB2 on cell proliferation and migration in endometrial carcinoma cells. A, B: Knockdown of NUCB2 mRNA level in Ishikawa (A) and Sawano (B) cells transfected with specific siRNAs for NUCB2 (siNUCB2 #1, 2) compared with those transfected with nonspecific control siRNA (siNC) (upper panel). NUCB2 expression was examined by real-time PCR analysis, and NUCB2 mRNA level was evaluated as the ratio of RPL13A mRNA level, and subsequently, relative NUCB2 mRNA level was summarized as a ratio (%) compared with siNC group. Knockdown of NUCB2 was confirmed at protein level by immunocytochemistry (lower panel). Bar = 25 μm, respectively. C, D: Relative cell proliferation of Ishikawa (C) and Sawano cells (D) were evaluated as a ratio (%) compared with that at day 0. E, F: Relative cell migration of Ishikawa (E) and Sawano (F) were evaluated as a ratio (%) compared with that of siNC group. * P < 0.05, ** P < 0.01 and *** P < 0.001 compared to siNC group, respectively.
NUCB2 in endometrial carcinoma

295

lian target of rapamycin (mTOR) pathway [24, 25], an important signal cascade in dysregulation of cell proliferation. However, to the best of our knowledge, effects of nesfatin-1 on phosphorylation of mTOR have not been examined in endometrial carcinomas. Therefore we treated Ishikawa and Sawano cells with nesfatin-1 and examined whether nesfatin-1 alters mTOR phosphorylation. As shown in Fig. 4D, mTOR protein was significantly phosphorylated after nesfatin-1 treatment in both Ishikawa and Sawano cells.

**Discussion**

This is the first study which examined the expression of NUCB2 in endometrial carcinoma tissues. We first showed that NUCB2 immunoreactivity was detected in 22% of endometrial carcinomas examined, and was increased compared to non-neoplastic endometrial glands. It has been reported that NUCB2 protein and its binding sites are expressed in mouse uterus, and play pivotal roles in the collapse and recovery of endometrium [26]. Honda et al. also found out that NUCB2 expression was differentially detected in endometriosis by Serial Analysis of Gene Expression (SAGE) methods [27]. These findings all suggest the importance of NUCB2 in endometrial disorders as well as normal endometrium.

In the present study, NUCB2 status was positively associated with Ki 67 LI. Ki 67 is well-established marker for evaluation of proliferation activity of endometrial carcinoma cells [28, 29]. We also demonstrated that knockdown of NUCB2 using specific siRNA resulted in decreased cell proliferation and migration property in Ishikawa and Sawano cells, and nesfatin-1, a derivative form of NUCB2 significantly stimulated cell proliferation and migration in Ishikawa cells.
cells. These findings are in good agreement with previous report by Suzuki et al., in which they reported NUCB2 promoted cell proliferation of breast cancer cell lines [17]. It is therefore considered that NUCB2 and/or nesfatin-1 are associated with aggressiveness of endometrial carcinomas by promoting proliferation and migration of endometrial carcinoma cells.

NUCB2 has several functional domains as well as proteolytic derivatives, and this may be the reason for the wide variety of cellular function of NUCB2. We demonstrated that knockdown of NUCB2 resulted in decreased cell proliferation and migration of both Ishikawa and Sawano cells, while increased cell proliferation and migration by nesfatin-1 was observed in Ishikawa cells but not in Sawano cells. These findings suggest that both proteolytic products and intracellular domains are involved in the protumorigenic roles of NUCB2. We demonstrated that nesfatin-1 stimulated mTOR phosphorylation, an important signal cascade in dysregulation of cell proliferation, differentiation, and survival of several types of human malignancies including endometrial carcinomas [24, 25], and that nesfatin-1 is therefore suggested to stimulate proliferation and migration of endometrial carcinoma cells partially by activating mTOR signaling. On the other hand, NUCB2 has been reported to interact with postmitotic growth suppressor neddin and involved in survival and death of postmitotic cells by controlling Ca\(^{2+}\) homeostasis in the cytoplasm [12], while Ca\(^{2+}\) homeostasis is implicated not only in non-neoplastic cells but cancer cells [30]. In 3T3-L1 adipocytes, knockdown of NUCB2 increased the intracellular Ca\(^{2+}\) levels, followed by increased serine/threonine protein phosphatase 2A (PP2A) activity [31]. PP2A stabilizes several tumor suppressors such as p53 and p107, and PP2A itself is also recognized as a tumor suppressor in human malignancies including endometrial carcinomas [31, 32]. The in vitro experiments conducted in our study are preliminary, and we could not identify the functional domains that are responsible for the cell proliferation and migration property. However, our experiments serve as a starting point for the clarification of biological functions of NUCB2 in endometrial carcinoma, and further examinations are required to explore the molecular mechanisms by which NUCB2 promotes the progress of endometrial carcinomas.

However, contradictory findings have also been also reported. In HO-8910 ovarian epithelial carcinoma cells, nesfatin-1 significantly suppressed ovarian carcinoma cell proliferation and promoted apoptosis [19], and similar findings have been reported in H295R adrenocortical carcinoma cells [20]. This inconsistent finding may be partially explained by the diversity of phosphorylation signal transduction. For example, in the report by Xu et al., which reported tumor-suppressive roles of nesfatin-1, nesfatin-1 decreased mTOR phosphorylation [19]. On the other hand, in this study, we demonstrated significant phosphorylation of mTOR following nesfatin-1 treatment in Ishikawa endometrial cancer cells. Similarly, nesfatin-1 is reported to decrease ERK\(_{1/2}\) phosphorylation and exert anti-proliferative effects on H295R adrenocortical carcinoma cells [20], while it is also reported that NUCB2 is a positive mediator of EGF-stimulated ERK phosphorylation [33]. It is therefore suggested that the biological function of NUCB2 may vary among tissues, and further examinations are therefore needed to clarify the molecular mechanism associated with the diverse roles of NUCB2.

To the best of our knowledge, this is the first study to demonstrate the association between NUCB2 immunoreactivity and clinical outcomes of endometrial carcinoma patients. NUCB2 status was significantly correlated with increased risk of recurrence and poor prognosis in endometrial cancer patients, and multivariate analysis revealed that NUCB2 status was an independent prognostic factor for both recurrence and cancer-specific survival of the patients. These findings are in good agreement with previous reports [17, 18]. Actually, Suzuki et al. demonstrated that immunohistochemical NUCB2 status as a potential prognostic predictor in breast carcinomas [17], and Zhang et al. also pointed out the possible link between NUCB2 mRNA level and recurrence of prostate carcinomas [18]. Immunohistochemical NUCB2 status is therefore considered a possible prognostic predictor of endometrial carcinomas.

We further examined prognostic significance of NUCB2 according to stage and histological grade, which were widely used for prognostic prediction, and demonstrated that NUCB2 status was still a prognostic marker for patients with early stage (stage I and stage II) and/or low-grade (grade 1 and grade 2) endometrial carcinomas. Although patients with early stage endometrial carcinomas had a relatively better prognosis, 10-17% of patients are considered to have a recurrence after surgery [34, 35], although the application of adjuvant therapy for these patients remains to be determined [2-6]. NUCB2 may thus serve as a useful biomarker independent of existing clinical and/or pathological parameters and therefore patients generally...
who are assumed to have a good prognosis (i.e. early stage and low histological grade) may also benefit from immunohistochemical NUCB2 evaluation.

It is well known that estrogen and progesterone play important roles in the development of endometrial carcinomas, especially endometrioid endometrial adenocarcinomas. Previously, we identified NUCB2 as an estrogen responsive gene in breast carcinoma cells. Moreover, Kim and Yang have pointed out the possibility that NUCB2 expression in mouse uterus is under the control of estrogens [26], and recently Chung et al. reported that NUCB2 expression in mouse pituitary gland was under the control of estrogen and progesterone [36]. However, in the present study, significant correlation was not detected between NUCB2 and ER or PR status. Previously, Nonogaki et al. found out that serotonin systems upregulate the expression of hypothalamic NUCB2 using mouse model [37], and Ramanjaneya et al. reported that NUCB2 protein was significantly increased by inflammatory cytokines, tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) in mouse fibroblast cell line, 3T3-L1 cells [38]. Thus, factors other than estrogens, which still remain to be elucidated, might be involved in the regulation of NUCB2 expression in endometrial carcinomas and contribute to the progression of endometrial carcinomas. Further studies are needed in order to clarify tissue-specific regulation of NUCB2 expression.

In summary, NUCB2 status was significantly correlated with cell proliferation activity of endometrial carcinoma cells, and was an independent prognostic factor for the patients. Subsequent in vitro studies demonstrated that NUCB2 significantly promoted cell proliferation and migration property in Ishikawa and Sawano cells. These findings suggest important roles of NUCB2 in the progress of endometrial carcinomas, and immunohistochemical NUCB2 status may contribute to identify high-risk patients with endometrial carcinomas.

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**Disclosure Information**

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Supplementary Fig. 1** Effects of conditioned medium from NUCB2-silenced endometrial carcinoma cells on proliferation and migration. Conditioned medium (CM) was collected from Ishikawa and Sawano cells in which NUCB2 expression was suppressed by siRNAs (siNUCB2 #1, 2). Proliferation assay (A: Ishikawa cells, and B: Sawano cells) and migration assay (C: Ishikawa cells, and D: Sawano cells) was performed by adding corresponding CM (10% v/v) to culture medium. Non-specific siRNA (siNC) was used as negative control.
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