*Original research papers*

# Inverse correlation between Skp2 and p27$^{Kip1}$ in normal endometrium and endometrial carcinoma

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**Abstract**

Cyclin-dependent-kinase (cdk) inhibitor, $p27^{Kip1}$ (p27), has been shown to participate in progestin-induced growth suppression of normal endometrial glands. To analyze the molecular mechanisms regulating p27 protein, we examined immunohistochemical expression of the $SCF^{Skp2}$ complex factors, i.e., Skp1, Cul1 and Skp2, and compared them with that of p27, steroid receptors and Ki-67. In normal endometrial glands, the expression of Skp2 was observed in the proliferative phase, whereas that of p27 was observed in the secretory phase. Cultured normal endometrial glandular cells showed that progesterone induced the down-regulation of Skp2 along with up-regulation of p27. In endometrial carcinomas, the inverse topological correlation between Skp2 and p27 was evident in 39/66 (59%) cases, and the expression of Skp2 showed a strong correlation with Ki-67. These findings suggest that the expression of $SCF^{Skp2}$ complex changes during the menstrual cycle in normal endometrium and the $SCF^{Skp2}$ ubiquitin-proteasome pathway may also work in endometrial carcinomas.

## Introduction

Normal endometrial glands and a subset of endometrial carcinomas express estrogen receptors (ER) and progesterone receptors (PR). Proliferation of these cells is stimulated by estrogen and inhibited by progesterone [1]. Recent research has revealed that cell growth is exquisitely controlled by cell cycle-regulators such as cyclins, cyclin-dependent kinases (cdks) and tumor suppressor gene products [2-4]. We previously reported that the expression of a cdk2 inhibitor, $p27^{Kip1}$ (p27), a negative regulator of the G1 phase of the cell cycle [5-8] is markedly increased in the glandular cells of normal endometrium during the secretory phase of the menstrual cycle in association with reduced expression of Ki-67 [9], and that p27 expression is increased by progesterone and actually involved in the growth suppression of cultured normal endometrial glandular cells and PR-positive endometrial carcinoma cells [10]. In our previous study, up-regulation of p27 protein by progestins was suggested to be regulated by post-translational mechanism [10]. However, the molecular machinery involved in controlling the intracellular level of p27 protein remains undetermined.

Recently, cell cycle-regulators have been reported to be degraded by ubiquitin-proteasome pathways [11, 12]. The SCF (Skp1-Cullin-F-box protein) complexes are the largest family of ubiquitin-protein ligases, and $SCF^{Skp2}$ is composed of Skp1 (S-phase kinase associated protein 1), Cul1 (cullin 1) and Skp2 [13, 14]. Skp1 serves as an adapter that links the F-box protein to Cul1 [15]. Cul1 is one of the cullin family proteins and acts as a scaffold protein of the complex [16]. Skp2 is a member of the F-box protein family, and functions as a p27-recognition subunit [15] . Thus, the $SCF^{Skp2}$ complex is regarded as a specific ubiquitin-ligase of p27 [17-19] and ubiquitinated p27 is then degraded by the proteasome [20]. However, there have been few reports on the expression of these factors in human endometrial tissues. Therefore, in the present study, we examined by immunohistochemistry the expression and localization of Skp1, Cul1 and Skp2 in normal endometrium. The expression and localization of these factors were also compared with those of p27, ER, PR, and a proliferation marker Ki-67. Based on the inverse topological correlation between p27 and Skp2, we examined in vitro the progesterone-induced change in the expression of

p27 and Skp2 using the primary culture of normal endometrial glandular cells. Finally, we also analyzed by immunohistochemistry the expression and localization of Skp1, Cul1 and Skp2, as well as their correlation with that of p27, ER, PR and Ki-67, in endometrial carcinomas.

## Materials and methods

*Histological materials*

Twenty-one samples of normal endometrium and 66 samples of endometrial carcinoma were selected from the pathology file of Shinshu University Hospital. The tissues of normal endometrium were obtained from patients aged 35-45 years who underwent hysterectomy for leiomyoma or carcinoma in situ of the uterine cervix. The tissues of endometrial carcinoma were obtained from patients aged 27-78 years who underwent hysterectomy. These specimens were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Serial sections of 3-μm thickness were made and processed for haematoxylin and eosin (HE) staining and for immunohistochemistry. In normal endometrium, endometrial dating was performed by judging an HE-stained slide according to the classification system of Noyes [21]. Of the 21 normal endometrial tissue specimens, 10 were in the proliferative phase, 4 were in the early secretory phase, 4 were in the mid secretory phase and 3 were in the late secretory phase. Of the 66 endometrial carcinomas, 38 were stage I, 7 were stage II, 16 were stage III, 5 were stage IV, and histologically, 31 were grade 1, 19 were grade 2, and 16 were grade 3, according to the FIGO (International Federation of Gynecology and Obstetrics) classification. Each tissue was used with the approval of the Ethics Committee of Shinshu University after obtaining written consent from the patients.

*Immunohistochemistry*

Indirect immunostaining was performed using antibodies against Skp1, Cul1, Skp2 and p27, which were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Ki-67 antibody was purchased from DAKO (Glostrup, Denmark). An anti-ER antibody that recognizes ER-α and an anti-PR antibody that recognizes both PR-A and PR-B were purchased from Immunotech (Marseille, France). Immunohistochemical staining was performed by the streptavidin-biotin-peroxidase complex method using a Histofine SAB-PO detector kit (Nichirei, Tokyo, Japan). Briefly, after deparaffinization in xylene and rehydration through graded alcohols, each section was treated by microwaving in 0.01 M citrate buffer (pH 6.0) for 15 minutes.

Endogenous peroxidase activity was blocked by 0.03% hydrogen peroxide in methyl alcohol for 30 minutes. Then, 10% normal goat (for rabbit primary antibodies) or rabbit (for mouse primary antibodies) serum was applied to avoid non-specific reactions. The sections were then incubated with specific primary antibodies [diluted 1:50 with phosphate-buffered saline (PBS)/bovine serum albumin (BSA) for anti-Skp1, anti-Cul1 and anti-Skp2 antibodies, 1:500 for anti-p27 and 1:100 for anti-ER, PR, and Ki67 antibodies] at 4°C overnight. After washing with PBS, biotinylated anti-mouse or rabbit IgG was applied for 60 minutes at room temperature. After washing with PBS, peroxidase-conjugated streptavidin solution was applied for 15 minutes, and staining was visualized by reaction with 0.05% 3'-3' diaminobenzidine (DAB). Light counterstaining was performed with haematoxylin.

*Interpretation of staining and statistical analysis*

In the present study, only nuclear staining for Skp1, Cul1, Skp2 and p27 was counted because these molecules function mainly in the nucleus [17-19]. The positivity of each staining was described as a positivity index (PI), which was the percentage of nuclear-positive cells in 500 cells from more than three high power fields. The PI was measured by two observers (T. M and T. S), and the mean value of the two observers was presented. For the statistical analysis, comparison of PI for the difference among menstrual phases, clinical stages and histological grades were made by Kruskal-Wallis rank test and Scheffe's test. A P value of less than 0.05 was considered significant. Correlations of PI among the factors were evaluated by Spearman's rank correlation. A P value of less than 0.05 was considered significant. A $\rho$ (correlation coefficient) value $>0.4$ or $<-0.4$ indicates a strongly positive or negative correlation. These analyses were made using StatView software (Abacus, Berkeley, CA).

*Cell culture and Western blotting*

1) Cultured normal endometrial glandular (NEG) cell: NEG cells were prepared and cultured with Ham F12+15% FCS as described previously [10]. The effect of progesterone (P4) on the expression of Skp2 and p27 in these cells was examined using

Western blotting. NEG cells ($1x10^6$) were dispersed in type IV collagen-coated 6-cm dishes and incubated with Ham F12+15% FCS for 24 hours. The serum was starved for 24 hours, and P4 was added daily at the concentration of $10^{-8}$ and $10^{-6}$ M and incubated for 7 days. The NEG cells after 7 days culture were confirmed to contain more than 86% of the cytokeratin-positive cells (data not shown). Medium was changed every 3 days. In addition, to confirm the specificity of the involvement of PR, the NEG cells was cultured for 3 days with daily addition of a PR antagonist, RU486 (Sigma, $10^{-7}$M), with P4 ($10^{-8}$M). After the cell culture, cells were collected and were subjected to Western blotting as previously described [10]. The filters were incubated with the same antibodies against Skp2 and p27 as used for imunohistochemistry. Then the filters were incubated in peroxidase-conjugated anti-mouse or rabbit IgG and the bound antibody was detected with an enhanced chemi-luminescence (ECL) system (Amersham, Buckinghamshire, UK). To further examine the functional correlation between Skp2 and p27 in NEG cells, the expression of Skp2 was suppressed using anti-sense oligo DNA, and the effect on p27 expression was evaluated. In brief, $4x10^5$ NEG cells were dispersed in 6 well plates with Ham's F12 with 15% FCS. Forty-eight hours after disperse, sense/anti-sense primers (anti-sense; cctgggggatgttctca, sense; ccgctcatcgtatgaca) were introduced at a final concentration of 1μM using Effectene transfection reagent Kit (QIAGEN). The cells were treated with transfection reagent only as a mock-transfection control. The cells were harvested 48 hours after the DNA introduction, and the expression of Skp2 and p27 proteins were evaluated by Western blotting. The density of the bands was quantified by densitometric analysis using a Quantity One Scan System (ATTO, Tokyo, Japan), and the results were given relative to the loading control (β-actin).

2) Permanent PR-transfectant of Ishikawa cell: A PR-positive endometrial carcinoma cell line, Ishikawa, was kindly provided by Dr. Nishida at Tsukuba University. Although Ishikawa cell is known to express PR (-B), the expression is reportedly weak. Therefore, we have established permanent PR-transfectants of Ishikawa cells to further clarify the involvement of PR. In brief, $5 \times 10^5$ Ishikawa cells were seeded in 6 cm dishes with

DMEM+10%FCS. Twenty-four hours after seeding, 0.9 μg of pSG5-PR-B transient expression vector (a kind gift from Dr. Kato K at Kyushu University with the permission from Dr. Chambon P at Pasteur University) and 0.1 μg of pcDNA3 were co-transfected using Effectene transfection reagent. For negative control, only pcDNA3 (1 μg) was transfected to the same cells. The transfection-positive colonies were selected using 800 μg/ml of G418 (Wako Pure Chemical, Osaka, Japan). A total of 5 permanent transfetant was isolated. The expression of PR was evaluated using reverse transcriptase-polymerase chain reaction (RT-PCR) as described below. The expression of Skp2 and p27 in the PR-transfected Ishikawa cells after the addition of P4 ($10^{-8}$M) with or without RU486 ($10^{-7}$M) was examined using Western blotting.

RT-PCR for PR-B

RT-PCR for PR-B was performed according to the previous study [22]. In brief, total RNA was isolated from each transfectrant using TRI zol (Invitrogen), and 1μg of RNA was reverse transcriptased using oligo-dT promers and RT PCR Kit (TaKaRa RNA PCR Kit Ver.3.0, Otsu, Japan). The products were then amplified using the same Kit and primers (sense; gcttcaagttagccaagaagagt, anti-sense promer; ctggaaattcaacactcagtg) according to the manufacturer's instruction.

## Results

*Normal endometrial glands*

The PIs of Cul1, Skp1, Skp2, p27, ER, PR and Ki-67 in the normal endometrial glands during the menstrual cycle are listed in Table 1. Nuclear expression of Cul1 and Skp2 was predominantly observed in the proliferative phase (Fig. 1a, c) and was markedly decreased in the secretory phase (Fig. 1e, g). The PIs of Cul1 and Skp2 were significantly different between the proliferative and mid-secretory phases; 60.3 and 1.5 (mean PI) for Cul1 ($P<0.05$), 18.6 and 0 for Skp2 ($P<0.05$), respectively. Nuclear expression of Skp1 was constantly observed in the glandular cells, and the PI was very high throughout the menstrual cycle (76.3±11.4 - 90.5±2.1). There was no significant difference of the PI between in the proliferative and secretory phases (Fig. 1b, f). The expression of ER, PR and Ki-67 was observed mainly in the proliferative phase, and was decreased in the secretory phase. Examination of the serial sections of normal proliferative endometrium revealed that Skp2-positive cells were positive for both Skp1 and Cul1, and these cells were also positive for Ki-67.

On the other hand, nuclear expression of p27 in the glandular cells of the functional layer was almost negative during the proliferative phase. In the secretory phase, however, the PI of p27-positive cells was increased to 53.5 in early, 87.8 in mid-, and 80.3 in late secretory phases (Fig. 1d, h). Examination of the serial sections revealed that p27-positive cells in the secretory phase were negative for Cul1 and Skp2, and also negative for Ki-67, indicating that inverse relationship between p27 and Ki-67 is associated with the change in the expression of Cul1 and Skp2 (Fig. 2). In addition, our further observation disclosed that several p27-positive glands were present in the basal layer of the endometrium even in the proliferative phase, and they were Skp2-negative and Ki-67-negative. In contrast, p27-negative glands in the adjacent functional layer were frequently positive for Skp2 and Ki-67. Thus, there was an inverse topological correlation between the expressions of Skp2 and p27 (Fig. 1i).

In cultured NEG cells, Western blot analysis showed that the expression of Skp2 was decreased under the treatment with progesterone (P4) in a dose-dependent fashion. In contrast, the expression of p27 was increased after the P4 treatment (Fig. 3a).

The addition of P4 also reduced the expression of Skp2 and induced the expression of p27, and these changes were suppressed by the addition of RU486 (Fig. 3b), indicating that these P4-induced reactions are mediated via PR. The introduction of anti-sense Skp2 to NEG cells suppressed the expression of Skp2, and was associated with increased expression of p27 (Fig. 3c).

*Endometrial carcinomas*

The PIs of Skp1, Cul1, Skp2, p27, ER, PR and Ki-67 in the endometrial carcinomas are summarized in Table 2. The PI of Skp1 was high irrespective of the histological grade of the tumor (Fig. 4a, e). The PIs of Skp2, p27 and Ki-67 were significantly higher in higher grade tumors ($P<0.05$) (Fig. 4c, d, e). In contrast, the PIs of ER and PR were decreased in tumors of higher histological grades ($P<0.05$, Table2). With respect to the FIGO stage, although the PI of p27 in stage III+IV tumors (31.3±32.0) was higher than that of stage I+II tumors (14.2±22.4), the difference did not reach a significant level. There were no marked differences in the PI of other factors according to the FIGO stage. With regard to correlations among the factors examined, the PI of Skp2 was strongly correlated with that of Ki-67 ($P<0.0001$, $\rho=0.742$, Fig. 5). A weak inverse correlation was observed between the PIs of Skp2 and ER ($P=0.0129$, $\rho=-0.308$) and between those of p27 and PR ($P=0.0383$, $\rho=-0.257$). There were no statistical correlations among the PIs of Cul1, Skp1, Ki-67 and p27 (data not shown).

On closer observation, the distribution of Skp2-positive cells tended to be topologically correlated with that of Ki-67-positive cells (Fig. 6a-f). Furthermore, the Skp2-positive cells were found in the regions consisted of p27-negative cells, indicating an inverse correlation between Skp2 and p27. Such an inverse topological correlation was observed in 39 (59%) of the 66 carcinomas; 10 (32%) of the 31 grade 1, 10 (53%) of the 19 grade 2 and 3 (19%) of the 16 grade 3 tumors, and tended to be more frequently found in areas showing glandular architectures rather than those with solid growth of the tumor cells. In the tumor cells positive for Skp2 and negative for p27, the expression of Cul1 was usually positive. In the remaining 27 carcinomas, such clear-cut inverse correlation was not evident because most tumor cells were negative for both

Skp2 and p27. In grade 3 carcinomas, both Skp2-positive cells and p27 positive cell was increased. Interestingly, in 3 cases of grade 3 carcinomas, Skp2-positive carcinoma cells were observed in the areas consisted of p27-positive cells, indicating co-expression of p27 and Skp2 (Fig. 6g, h). In areas positive for both Skp2 and p27, the expression of Cul1 tended to be decreased. Remaining 10 cases of G3 carcinomas did not show such topological correlation.

The effect of progesterone on the expression of Skp2 and p27 was also examined in endometrial carcinoma Ishikawa cells (Fig. 7). In pcDNA3-transfected (mock) Ishikawa cells, the addition of P4 resulted in only slight reduction of both Skp2 and p27 expressions. RU486 treatment showed no marked changes. In PR-transfected Ishikawa cells, P4 treatment resulted in the marked reduction of Skp2 expression, and this reduction was rescued by RU486. However, contrary to NEG cells, P4 treatment induced only slight increase in the expression of p27.

**Discussion**

The present study revealed the expression patterns of Skp1, Cul1 and Skp2, which organize the $SCF^{Skp2}$ complex that is known to be involved in protein ubiquitination, in normal human endometrium. Among the 3 proteins, Skp1 was constantly expressed in the glandular cells throughout the menstrual cycle. A previous study also showed that the mRNA and protein expression of Skp1 is stable in the rat brain throughout life [23]. Accordingly, Skp1 is considered to have the nature of a "housekeeping" gene. In contrast, the expression of Cul1 and Skp2 in normal endometrial glandular cells showed cyclic changes; it was stronger in the proliferative phase than in the secretory phase. The expression of Cul1 has been reported to be enhanced by c-Myc [24], which has reportedly been expressed in the nucleus of normal proliferative endometrium [25]. However, the mechanism of down-regulation of Cul1 in the secretory phase remains unclear. Skp2 was originally identified as a S-phase associated protein [26], and its transcription was reportedly up-regulated by GA-binding protein that is considered as an ETS-related transcription factor involved in various biological events including cell proliferation [27-30]. Therefore, it is reasonable that immunoreactivity for Skp2 was observed in the glandular cells of normal proliferative endometrium. The present study also indicated the cyclic changes in the expression of ER, PR and Ki-67, and our results correlated well with the previous reports [31, 32]. To our knowledge, this is the first report to demonstrate the cyclic changes in the expression of Cul1 and Skp2 in normal human endometrium. Since the positivity index (PI) of Skp2 was much lower than that of Cul1, the expression of Skp2 may be more essential for the function of $SCF^{Skp2}$ complex in normal endometrium.

Our study also showed a clear-cut inverse pattern of expressions between Skp2 and p27 in normal endometrium. This relationship was also observed by our in vitro study using anti-sense Skp2 oligo DNA in NEG cells, which resulted in the up-regulation of p27. In the glandular cells of the functional layer, Skp2 was expressed only in the proliferative phase, whereas p27 was mainly in the secretory phase. These findings suggest that progesterone up-regulates the protein level expression of p27 via down-regulation of Skp2 during the secretory phase. To address this hypothesis, we

examined in vitro the effect of progesterone, and found that the expression of Skp2 is decreased along with up-regulation of p27 under the treatment with progesterone in NEG cells. In addition, the progesterone-induced down-regulation of Skp2 and up-regulation of p27 was diminished by the treatment with RU386, suggesting that this reaction was mediated through PR. These findings indicate that progesterone induces down-expression of Skp2 which leads to the intracellular accumulation of p27, possibly via decreased degradation of p27 protein. However, even in the proliferative phase, several p27-positive glands were present in the basal layer of the endometrium, and they were Skp2-negative. Those findings suggest that some parts of protein level of p27 might be regulated by progesterone independent pathway. Since the Skp2-positive glandular cells were usually positive for both Skp1, Cul1, and Ki-67, it is likely that $SCF^{Skp2}$ complex is actually involved in the cell proliferation of normal endometrium.

In endometrial carcinomas, the inverse topological correlation between the expressions of Skp2 and p27 was clearly identified in 59% of endometrial carcinomas. Similar relationship between these 2 molecules has also been reported in oral squamous neoplasms [33] and astrocytic gliomas [34]. In our series of endometrial carcinoma, such inverse correlation tended to be more frequent in well- or moderately differentiated carcinomas possessing glandular architectures as observed in grade 1 (32%) and grade 2 (53%) tumors than those with grade 3 (19%) tumors. In these tumors showing the inverse correlation between Skp2 and p27, p27-positive tumor cells were negative for Ki-67, suggesting that p27 may act as a cell growth inhibitor. In addition, examination of serial sections revealed that Skp2-positive carcinoma cells were usually positive for both Skp1 and Cul1, and negative for p27. These findings suggest that the function of $SCF^{Skp2}$ complex is preserved in a subset of endometrial carcinomas. It should be noted that most of our patients with endometrial carcinoma were postmenopausal, and therefore, the expression of Skp2 is considered under the non-hormonal regulation. A recent report showed that the PI3-kinase/PTEN pathway may be involved in the control of Skp2 expression [35]. Additionally, the forkhead transcription factor FOXO1, a downstream target of phosphatidylinositol-3-kinase/Akt signalling pathway, has been reported to regulate p27 expression in normal endometrium and loss of FOXO1

promotes uncontrolled cell proliferation and increases susceptibility to genotoxic insults [36].

In our in vitro study using endometrial carcinoma cell lines, the mock Ishikawa cells did not show P4-induced down-regulation of Skp2 or up-regulation of p27, possibly due to the limited expression of PR. In the PR-transfected Ishikawa cells, P4 treatment reduced the expression of Skp2, and the reduction was rescued by RU486, indicating that this reaction was mediated via PR. This finding was consistent with that observed in NEG cells. Contrary to our expectation, P4-induced up-regulation of p27 was not evident in PR-tranfected Ishikawa cells. However, it is noteworthy that the expression of p27 was observed even when the basal expression of Skp2 was present in the absence of P4 in NEG cells as shown in Fig. 3a, suggesting the possible involvement of other mechanisms for p27 degradation. More importantly, the P4-induced up-regulation of p27 was not observed in endometrial carcinoma cells even when the expression of Skp2 was suppressed by P4. Recent studies have revealed that p27 was also degraded by KPC (Kip1 ubiquitination-promoting complex)-dependent ubiquitination [37] or heat shock protein (HSP)27-dependent mechanisms[38]. In vivo, the tumor cells were positive for both Skp2 and p27 in our 3 cases of poorly-differentiated endometrial carcinomas, implying the alteration in the mechanisms regulating the expression of Skp2 or p27 or both [39]. Collectively, the regulation of p27 protein in endometrial carcinoma cells may depend more on other mechanisms such as KPC and HSP27 [37, 38] compared with normal endometrial glands. Therefore, further studies are needed to clarify the mechanism of p27 protein regulation in this malignancy.

In endometrial carcinomas, the PI of Skp2 was strongly correlated with the cell proliferation as shown by the expression of Ki-67, and was also significantly higher in grade 3 (31.6±23.2) than in grade 1 (11.8±11.5) tumors. This is consistent with previous reports that expression of Skp2 was correlated with higher histological grade in breast carcinoma [40] and with the enhanced proliferation in malignant lymphomas [41]. Interestingly, in our series of endometrial carcinoma, the PI of p27 was also significantly higher in grade 3 (38.4±33.9) tumors than in grade 1 (12.6±24.9) or grade

2 (15.3±13.9) tumors. Such unexpectedly high expression of p27 has also been reported in other malignancies; breast carcinoma cells with higher growth potential have been reported to express higher levels of p27 protein [42]. In uterine cervical carcinomas, we have recently found that elevated expression of p27 was associated with increased growth activities, and our in vitro studies suggested the insufficient function of p27 in malignant tumors [43]. Paradoxical overexpression of p27 in human malignancies is an interesting issue which needs further clarification.

In conclusion, cyclic change in the expression of Skp2 organizing the $SCF^{Skp2}$ ubiquitin-proteasome pathway is involved in the progesterone-induced expression of p27, regulating the cell growth of normal endometrial glands. The function of the $SCF^{Skp2}$ complex is suggested to be preserved in a subset of endometrial carcinomas. Further studies are needed to clarify the hormonal and other mechanisms regulating the expression of Skp2 in normal and malignant endometrial tissues.

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**Declaration of interest:** We herby declare that all statements made throughout this manuscript are of our own knowledge and are accurate. The information contained therein is presented as a true representation of the data. There are no financial conflicts of interest with any organization about this work.

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**Table 1** Positivity index (PI) of the Skp1, Cul1, Skp2, p27, ER, PR and Ki-67 in normal endometrial glands

| Factor | Proliferative phase | Early secretory phase | Mid-secretory phase | Late secretory phase |
|---|---|---|---|---|
| Skp1 | 84.8±5.0 | 90.5±2.1 | 81.3±8.1 | 76.3±11.4 |
| Cul1 | 60.3±15.8* | 0.0 | 1.5±2.1 | 1.3±2.5 |
| Skp2 | 18.6±5.8* | 0.0 | 0.0 | 0.0 |
| p27 | 0.2±0.3* | 53.5±32.4 | 87.8±9.1 | 80.3±11.8 |
| ER | 96.1±4.8* | 31.3±11.7 | 0.0 | 0.0 |
| PR | 98.9±1.5** | 85.3±19.5 | 12.6±8.2 | 0.0 |
| Ki-67 | 24.5±6.9* | 2.2±1.8 | 0.1±0.3 | 0.0 |

Each number is the mean± standard deviation

*: significantly different from PIs in each of the secretory phases. ($P<0.05$)

**: significantly different from PIs in the mid- and late secretory phases. ($P<0.05$)

**Table 2** Positivity index (PI) of Skp1, Cul1, Skp2, p27, ER, PR and Ki-67 in endometrial carcinomas

| Factor | Total cases | Grade 1 | Grade 2 | Grade 3 |
|---|---|---|---|---|
| Skp1 | 82.5±13.5 | 82.1±12.7 | 80.9±15.0 | 85.2±13.7 |
| Cul1 | 19.1±31.0 | 17.1±29.5 | 7.4±19.0 | 36.8±38.6[b] |
| Skp2 | 19.2±16.9 | 11.8±11.5 | 20.7±11.6 | 31.6±23.2[a] |
| p27 | 19.6±26.8 | 12.6±24.9 | 15.3±13.9 | 38.4±33.9[a,b] |
| ER | 29.1±27.7 | 35.9±26.9 | 25.9±27.6 | 19.9±27.8[a] |
| PR | 45.2±32.2 | 51.3±28.6 | 44.1±33.9 | 34.6±35.8[a] |
| Ki-67 | 18.1±14.9 | 12.6±11.5 | 19.5±8.6 | 27.2±21.3[a] |

Each number is the mean± standard deviation

a: significantly different from PI in grade 1 ($P<0.05$)

b: significantly different from PI in grade 2 ($P<0.05$)

**Figure Legends**

**Fig. 1.** Immunostaining of Skp1, Cul1, Skp2 and p27 in the normal endometrium (a-d; serial sections of the proliferative phase, e-h; serial sections in the early secretory phase, a and e; Cul1, b and f; Skp1; c and g; Skp2; d and h; p27). Nuclear expression of Cul1 and Skp2 was observed in the proliferative phase (a and c), and markedly reduced in the early secretory phase (e and g). Skp1 expression was observed irrespective of the menstrual cycle (b and f).The expression of p27 was nearly negative in the proliferative phase (d), and markedly increased in the early secretory phase (h). (x200) The graph shows the summary of the positivity index (PI) of Skp1, Cul1, Skp2, p27, ER, PR and Ki-67 in the normal endometrium during the menstrual cycle (i). An inverse correlation between Skp2 and p27 was noted.

**Fig. 2.** Expression of p27 (a), Skp2 (b) and Ki-67 (c) in serial sections of the normal proliferative endometrium. Arrowhead; p27-positive gland, arrow; p27-negative gland. An inverse correlation between the spatial expression patterns of p27 and Skp2, and a positive correlation between those of Skp2 and Ki67, were observed. A p27-positive gland was observed in the basal layer (arrowhead), and it was Skp2-negative and Ki-67-negative. In contrast, Skp2-positive glands were observed in the adjacent functional layer (arrow), and they were Ki-67-positive and p27-negative.

**Fig. 3.** Expression of Skp2 and p27 in cultured normal endometrial glandular (NEG) cells (a-c) using Western blotting. a: Treatment of NEG cells with progesterone reduced the expression of Skp2 in a dose dependent fashion, whereas it increased the expression of p27. b: Addition of P4 ($10^{-8}$M) reduced the expression of Skp2 and induced the expression of p27, and these changes were abolished by the addition of RU486. c: Transfection of anti-sense Skp2 oligo DNA induced the expression of p27(Mock: control, S; sense, AS; anti-sense).

**Fig. 4.** Immunostaining of Skp1, Cul1 and Skp2 in endometrial carcinomas. (a-c are serial sections.) a; Skp1 in grade 1, b; Cul1 in grade 1, c; Skp2 in grade 1, d; Skp2 in

grade 3 carcinoma. Nuclear staining of each factor is observed. (x150) The graph (e) shows the summary of the positivity index (PI) of Skp1, Cul1, Skp2, p27, ER, PR and Ki-67 in endometrial carcinomas according to the histological grade. Results are expressed as mean values.

a: The PI of grade 3 tumors was significantly different from that of grade 1 tumors ($P<0.05$).

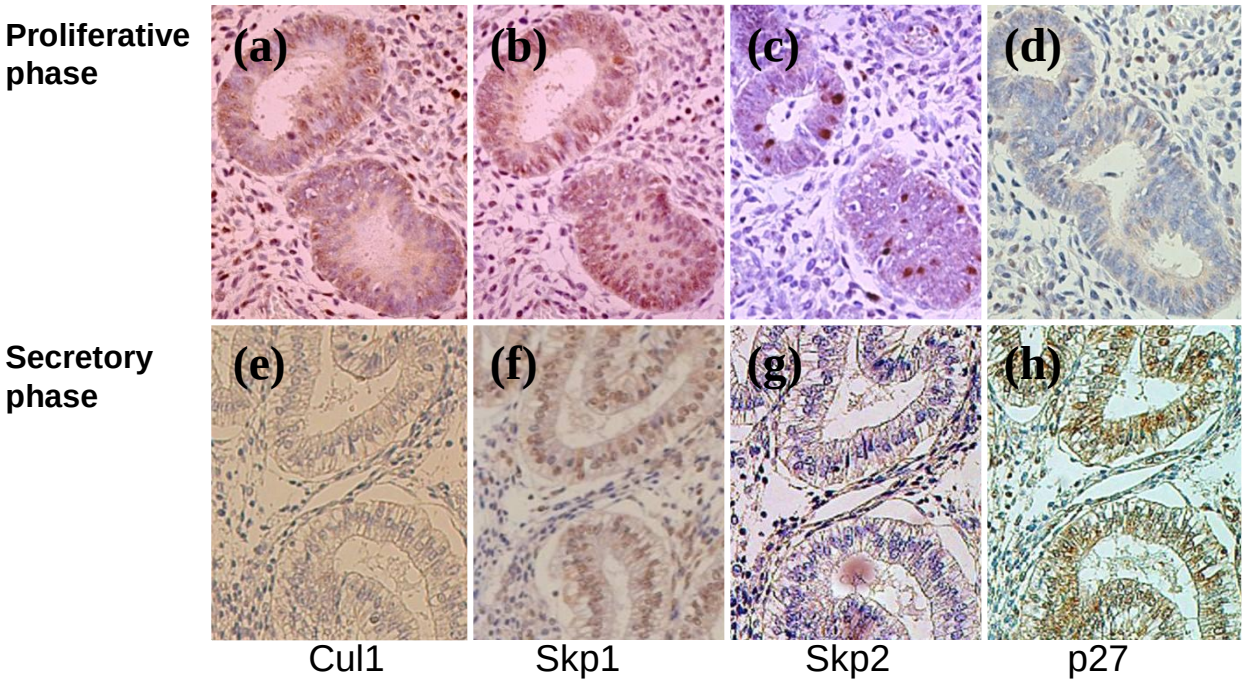
b: The PI of grade 3 tumors was significantly different from that of grade 2 tumors ($P<0.05$).

**Fig. 5.** Scatter plot of the positivity index (PI) of Skp2 and Ki-67 in endometrial carcinomas, showing a strong correlation between Skp2 and Ki-67 ($P<0.0001$, $\rho=0.742$).

**Fig. 6.** Expression of p27 (a and d), Skp2 (b and e) and Ki-67 (c and f) in serial sections of grade 1 (a-c) and grade 2 (d-f) endometrial carcinomas. p27-positive areas are indicated by arrows, and p27-negative areas by arrowheads. Spatially, Skp2 expression was inversely correlated with p27 expression, and positively correlated with Ki-67 expression. (a-c; x150, d-e; x250). In contrast, expression of p27 (g) and Skp2 (h) in serial sections of a grade 3 endometrial carcinoma showed co-expression of p27 and Skp2 in several cells. (x250)

**Fig. 7.** Expression of Skp2 and p27 in Ishikawa cells using Western blotting. In pcDNA3-transfected (mock) Ishikawa cells, P4 treatment resulted in slight reduction of both Skp2 and p27 expressions. RU486 treatment did not show significant changes. In PR-transfected (pSG5-PR-B) Ishikawa cells, P4 treatment resulted in the marked reduction of Skp2 expression, and this reduction was rescued by RU486 treatment, however, P4 treatment did not show apparent up-regulation of p27. The density of the p27 and skp2 bands is quantified by densitometric analysis. Data are presented after normalization with the β-actin bands. *$p<0.05$.

Fig. 1


Proliferative phase
Secretory phase
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
Cul1
Skp1
Skp2
p27

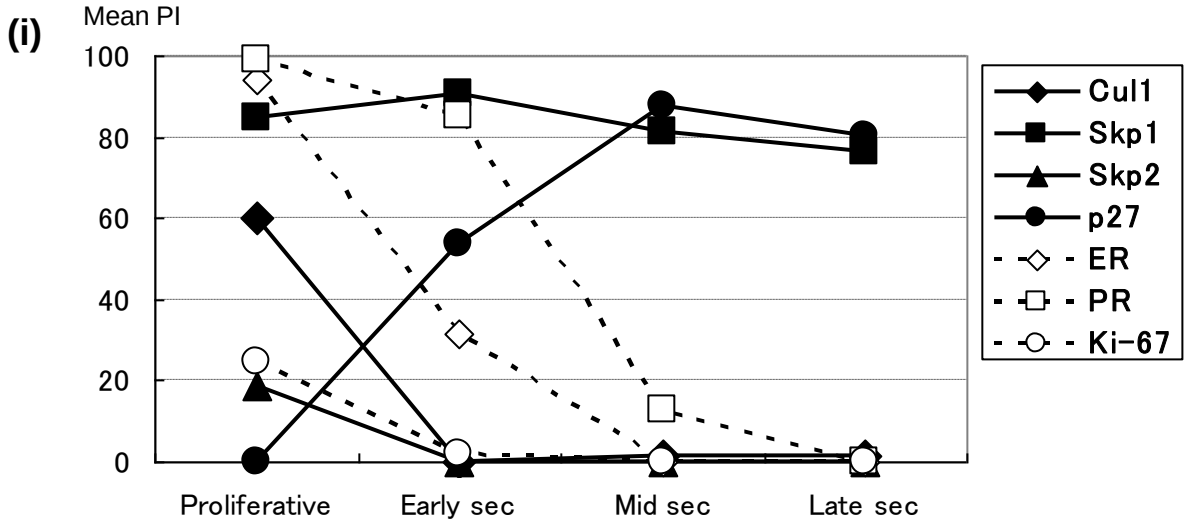

(i)
Mean PI
100
80
60
40
20
0
Proliferative
Early sec
Mid sec
Late sec
Cul1
Skp1
Skp2
p27
ER
PR
Ki-67

Fig. 2

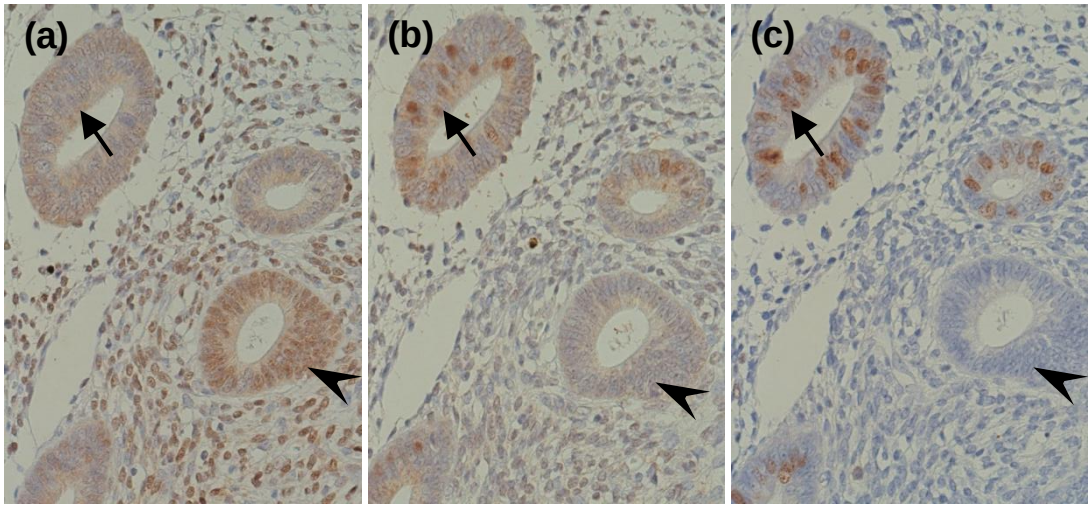
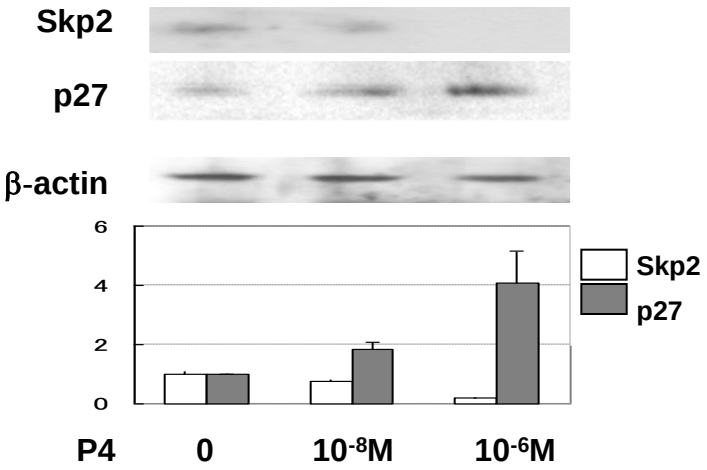


Fig. 3

a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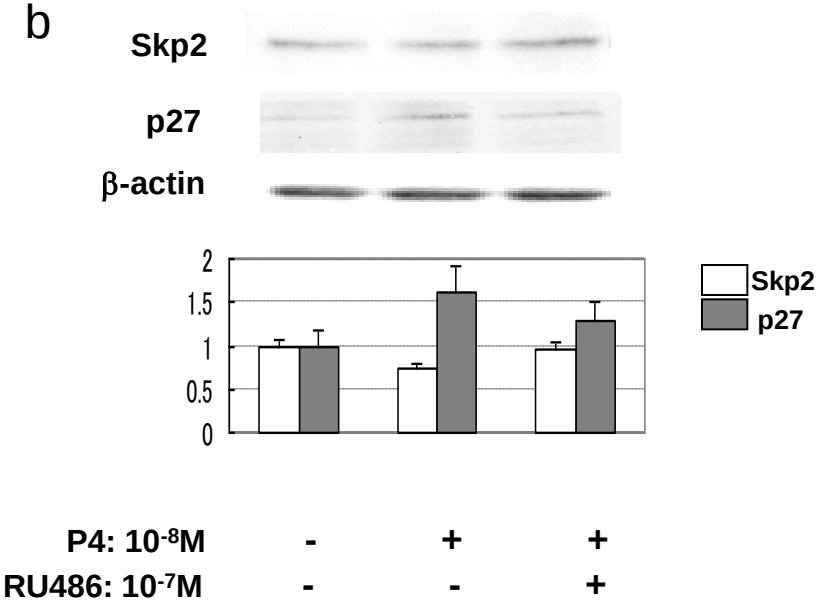
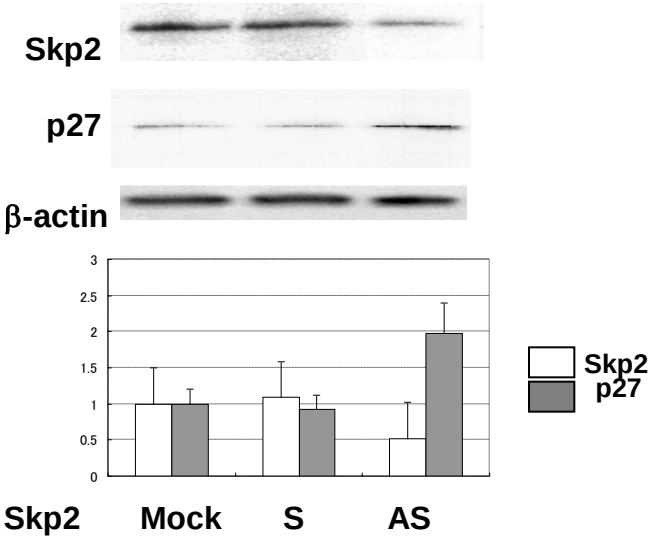
β-actin
6
4
2
0
Skp2
p27
P4
0
10-8M
10-6M

b


Skp2
p27
β-actin
2
1.5
1
0.5
0
Skp2
p27
P4: 10-8M - + +
RU486: 10-7M - - +

c


Skp2
p27
β-actin
3
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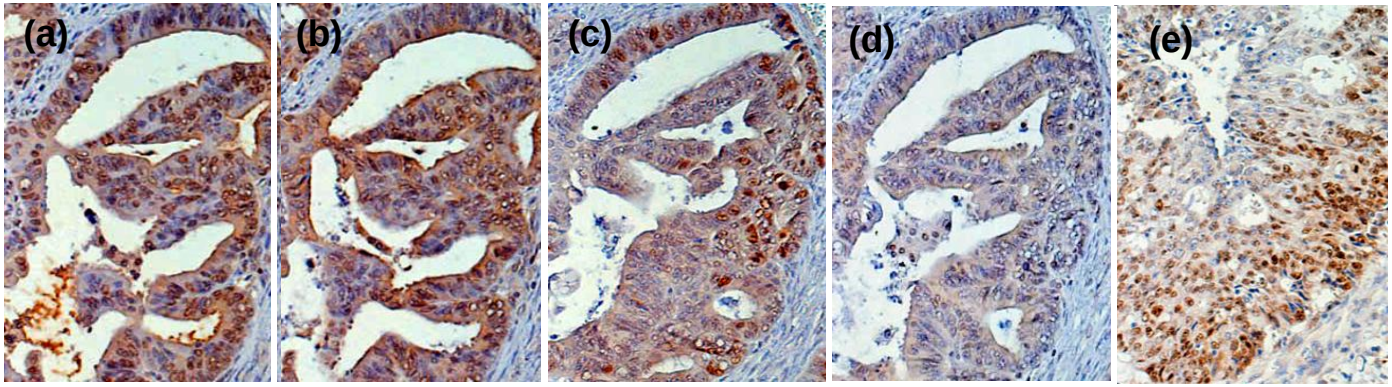
2
1.5
1
0.5
0
Skp2
p27
Skp2 Mock S AS

Fig. 4




SKp1/grade1 Cul1/grade1 Skp2/grade 1 p27/grade1 Skp2/grade 3

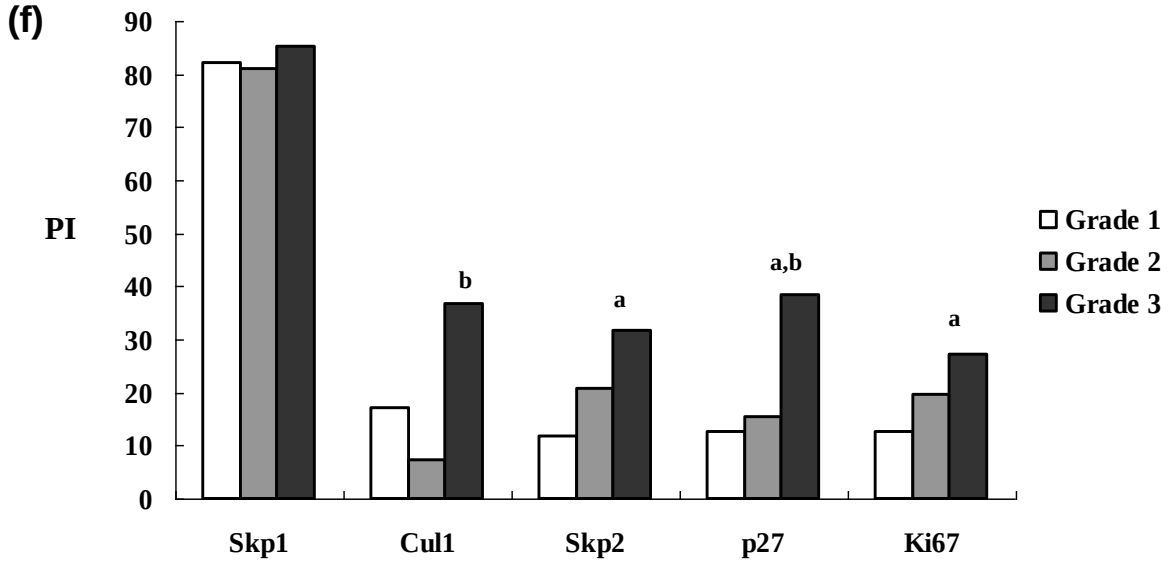


Fig. 5

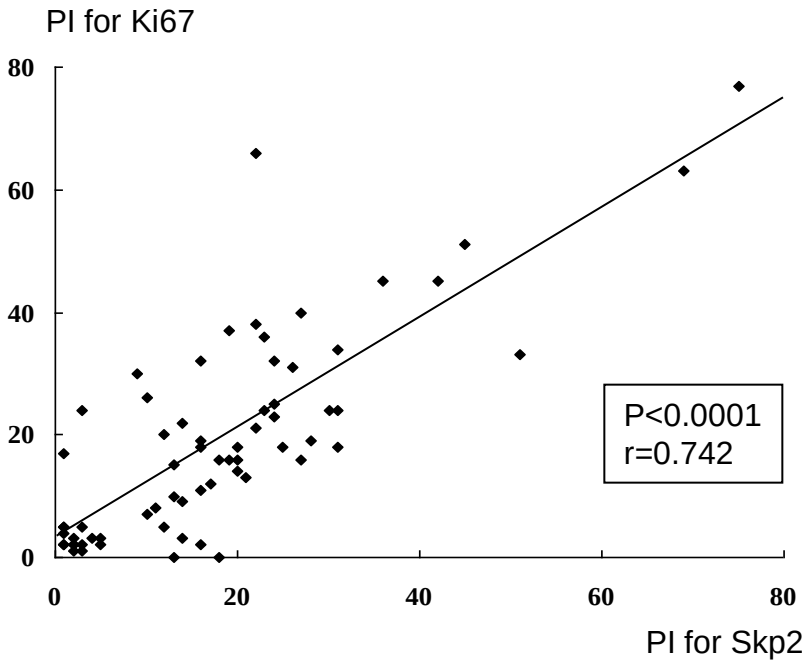
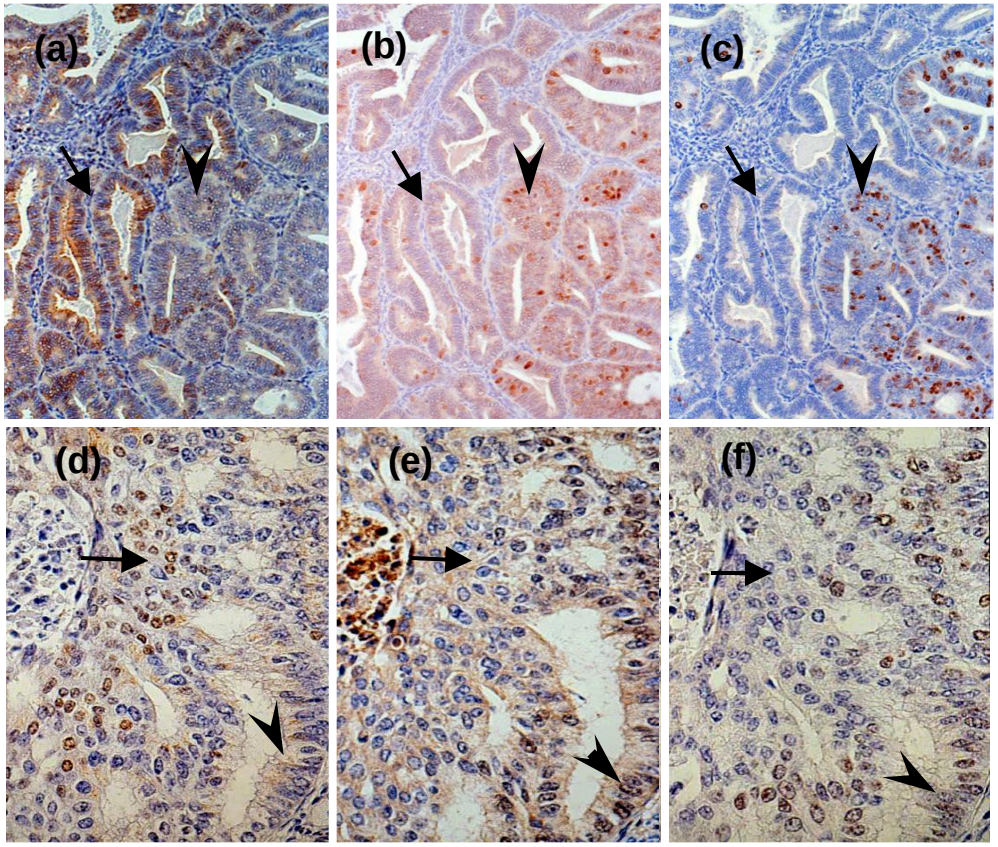


Fig.6



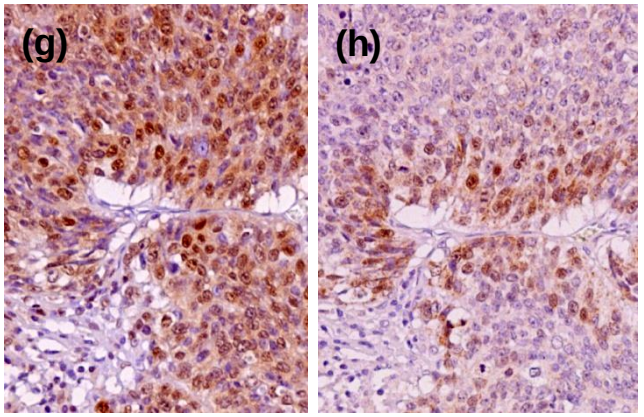
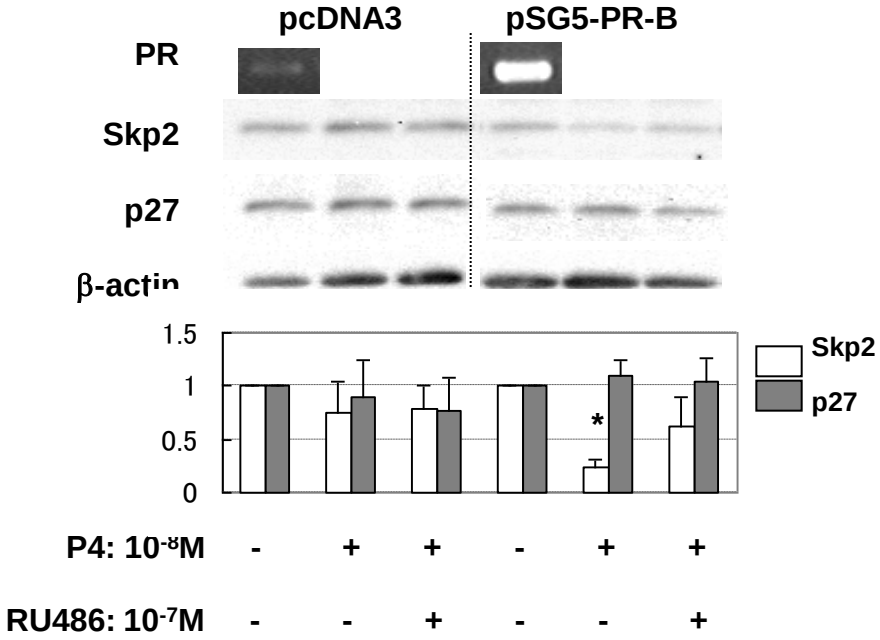



Fig.7


pcDNA3
pSG5-PR-B
PR
Skp2
p27
β-actin
1.5
1
0.5
0
*
Skp2
p27
P4: 10-8M - + + - + +
RU486: 10-7M - - + - - +