

**Cell proliferation effect of GnRH agonist on pathologic lesions of women with endometriosis, adenomyosis and uterine myoma**

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**Running Title:** Effect of GnRHa on cell proliferation in reproductive diseases

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## **Abstract**

**Background:** We recently demonstrated the effect of gonadotropin-releasing hormone agonist (GnRHa) on tissue inflammation, angiogenesis and apoptosis in endometriosis, adenomyosis and uterine myoma. Here, we investigated expression of GnRH receptors (GnRHR) and effect of GnRHa on the proliferation of cells derived from endometria and pathologic lesions of women with these reproductive diseases.

**Methods:** Biopsy specimens were collected from pathologic lesions and corresponding endometria of 35 women with pelvic endometriosis, 45 women with ovarian endometrioma, 35 women with adenomyosis, 56 women with uterine myoma during laparoscopy or laparotomy. The gene and protein expressions of GnRHR in eutopic/ectopic cells and tissues were examined by reverse transcriptase-polymerase chain reaction and immunohistochemistry. The immunoreactivity pattern of GnRHR expression in respective tissue was analyzed by quantitative-histogram (Q-H) scores. The exogenous effect of GnRHa on cell proliferation was examined by 5-Bromo-2-deoxyuridine incorporation assay. The Ki-67-immunoreactive cell proliferation index was analyzed in biopsy specimens derived from GnRHa-treated and -non-treated women.

**Results:** GnRH receptors, type I and type II, mRNAs and GnRHR proteins were expressed in eutopic endometria and pathologic lesions derived from women with endometriosis, adenomyosis and uterine myoma. GnRHR expression was the highest in the menstrual phase when compared with other phases of the menstrual cycle. The highest Q-H scores of GnRHR immunoreaction were found in blood-filled opaque red lesions than in other peritoneal lesions. The exogenous treatment with GnRHa significantly suppressed the proliferation of cells derived from respective endometria and pathologic lesions when compared with GnRHa-non-treated cells.

**Conclusion:** Local tissue expressions of GnRH receptor were detected in endometriosis, adenomyosis and uterine myoma. In addition to hypo-estrogenic effect, direct anti-proliferative effect of GnRHa may be involved in the regression of these reproductive diseases with consequent remission of clinical symptoms.

**Key Words:** GnRH receptor / GnRH agonist / cell proliferation / reproductive diseases

## **Introduction**

With the advent of isolation and synthesis of gonadotropin-releasing hormone (GnRH) by Schally (1971) in the early 1970s, interest in the clinical application of GnRH agonist (GnRHa) has grown. Now in clinical practice, GnRHa has been used for the medical treatment of prostate cancer, precocious puberty, endometriosis, adenomyosis and uterine myoma. Traditionally, the effect of GnRHa is mediated by competitive down regulation of pituitary GnRH receptors (GnRHR), causing a state of hypo-estrogenemia resulting in the resolution of pain symptoms and regression of diseases.

Endogenous GnRH (GnRH I and GnRH II) as well as exogenous GnRHa have been demonstrated to exert anti-proliferative and apoptotic effects on cultured endometriotic cells and some cancer cells derived from reproductive organs (Borroni et al., 2000; Imai et al., 2000; Tang et al., 2002; Limonta et al., 2003; Morimoto et al., 2005; Khan et al., 2009). The response of this hormonal medication to reproductive diseases is variable depending on the type of the medication, patients background and GnRH receptor-ligand binding affinity for individual cells or tissues (Qayum et al., 1990; Limonta et al., 1992; Emons et al., 1993; Borroni et al., 2000). Information regarding multiple biological functions of GnRHa in peripheral tissues of women with different

reproductive diseases was limited. We recently demonstrated changes in tissue inflammation, angiogenesis and apoptosis in endometriosis, adenomyosis and uterine myoma after GnRHa treatment (Khan et al., 2010). However, studies on the expression of GnRHR in peripheral tissues and direct cell proliferation effect of GnRHa in women with these reproductive diseases were not well described.

Therefore, we investigated three main issues in this study. Firstly, we examined gene and protein expression of GnRHR in isolated cells and intact tissues derived from women with endometriosis, adenomyosis and uterine myoma. Secondly, we examined cell proliferation effect of exogenous GnRHa on cells derived the respective endometria and pathologic lesions of women with these reproductive diseases. Thirdly, we investigated in vivo pattern of changes in cell proliferation in the endometria and pathologic lesions derived from GnRHa-treated and -non-treated women.

## **Material and Methods**

*Subjects.* The subjects in this study were women of reproductive age. From February 2004 to June 2009, biopsy specimens were collected from a total of 30 control women, 45 women with ovarian endometrioma, 35 women with adenomyosis and 56 women with uterine myomas who underwent hysteroscopy, laparoscopy or laparotomy during this period. All these women were admitted to our hospital with the complaint of abnormal genital bleeding, hypermenorrhoea or anemia with or without associated complaint of dysmenorrhea or pelvic pain. A fraction of these study groups had variable coexistent lesions of pelvic endometriosis. We also separately studied 35 women with biopsy proven pelvic endometriosis. All women with pelvic and ovarian endometriosis, adenomyosis and uterine fibroids were diagnosed by ultrasonography and magnetic resonance image before operation or by laparoscopy and subsequently confirmed by histology. The phases of the menstrual cycle in women without hormonal therapy was determined by histological dating of eutopic endometria samples taken simultaneously with peritoneal lesions, endometrioma, adenomyoma and nodules.

The staging and morphological distribution of peritoneal lesions of

endometriosis were based on the revised classification of the American Society for Reproductive Medicine (r-ASRM) (1997). As we described recently (Khan et al., 2004a), peritoneal lesions were categorized according to the color appearance of pelvic endometriosis. Biopsy specimens from each of these peritoneal lesions were collected for subsequent experimental analysis. All biopsy specimens were collected in accordance with the guidelines of the Declaration of Helsinki and with the approval of the Nagasaki University Institutional Review Board. An informed consent was obtained from all women.

***Biopsy specimens.*** Biopsy specimens were collected from the cyst wall, adenomyotic lesion, myoma nodule, autologous myometria, and respective endometria derived from women with endometrioma, adenomyosis and uterine myoma during operation. Endometrial samples were collected from all women with and without GnRHa therapy. We also collected biopsy samples from the eutopic endometria, ectopic endometria and adjacent peritoneum of women with pelvic endometriosis and control women. All collected biopsy specimens were prepared for formalin-fixed paraffin-embedded tissue blocks for subsequent histopathological and

immunohistochemical study.

***Isolation of endometrial cells.*** Isolation and culture of endometrial stromal cells and epithelial cells derived from the eutopic/ectopic endometria of women with endometriosis, adenomyosis and uterine myoma were processed as described previously (Koga et al., 2001; Khan et al., 2005a, 2005b, 2005c, 2008). Briefly, the tissues were minced into small pieces and incubated in DMEM/F-12 containing collagenase type I (2.5 mg/ml) (Sigma, St Louis, MO, USA) and deoxyribonuclease I (15 U/ml) (Takara, Tokyo, Japan) for 2-3 hours at 37°C. The resultant dispersed endometrial cells were separated by filtration through a 60-  $\mu$  m nylon cell strainer (Becton Dickinson and Co., Franklin lakes, NJ, USA). Endometrial epithelial glands that remained intact were retained by the strainer whereas dispersed stromal cells passed through the strainer into the filtrate.

Endometrial stromal cells in the filtrate were collected by centrifugation and resuspended in phenol-red free DMEM/F-12 containing 10% charcoal-stripped FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25  $\mu$  g/ml amphotericin B (all from Sigma). Stromal cells were seeded in a 100-mm culture plate and kept at 37°C in a



humidified 5%CO<sub>2</sub>-95% air atmosphere. At the first passage, cells were plated at a density of  $2 \times 10^5$  cells/ml. The cells reached confluence in 2 or 3 days and then were used for experiments.

The chocolate cyst linings of the ovaries were collected as the source of endometriotic tissues. Stromal cells from cyst wall were collected according to the method described in detail previously (Iwabe et al., 1998). We used stromal cells in a monolayer culture after the first passage.

Endometrial epithelial cells were collected by back washing the strainer with DMEM/F-12 containing 10% charcoal-stripped FBS, seeded in a 100-mm plate, and incubated at 37°C for 60 min to allow contaminated stromal cells to attach to the plate wall. The non-attached epithelial cells were recovered and cultured in the medium at a density of  $2 \times 10^5$  cells/ml. The cells, which reached confluence in 2 or 3 days, were used for experiments. The purity of stromal and epithelial cell preparation was more than 95%, as judged by positive cellular staining for vimentin and cytokeratin, respectively and negative cellular staining for CD45 (pan-leukocytes) and VWF (endothelial cells).

***Isolation of smooth muscle cells.*** Biopsy specimens from each of the

adenomyotic lesion and myoma nodule of GnRHa-treated and -non-treated women with adenomyosis and uterine myoma were collected and smooth muscle cells were isolated in primary culture as described previously (Rossi et al., 1992).

Briefly, fresh biopsy specimens collected in sterile medium were rinsed with PBS to remove blood cells. The tissues were cut into small pieces and digested in 2% collagenase (Sigma, St Louis, MO, USA) with DMEM and incubated in a shaking water bath at 37°C for 3-6 hours. Isolated smooth muscle cells were collected by centrifugation at 460g for 5-10 minutes, washed several times with DMEM containing 1% antibiotic-antimycotic solution (Sigma) and cultures in 75cm<sup>2</sup> flask in different aliquots. The isolated smooth muscle cells were grown as monolayers in 75cm<sup>2</sup> flask at a density of 2x10<sup>5</sup> cells per flask. The cells were maintained in pheno-red free DMEM supplemented with charcoal-stripped 10% FBS and 1% antibiotic-antimycotic solution for 6 days at 37°C in a humidified culture atmosphere of 5% CO<sup>2</sup>-95% air. The purity of smooth muscle cells was more than 95%, as judged by positive cellular staining for desmin or alpha-smooth muscle actin and negative cellular staining for vimentin, cytokeratin, CD45 and VWF. The smooth muscle cells were seeded after the third

passage at  $2 \times 10^4$  cells per well in a 96 well culture (Greiner) plate for cell proliferation study.

***Gene expression of GnRH receptor.*** Total RNA was isolated using ISOGEN method (Molecular Research Center, Tokyo) according to the manufacturer's protocol. RNA ( $1 \mu\text{g}$ ) was added to reverse the transcription reaction (RT-PCR), and cDNA ( $1 \mu\text{L}$ ) was subjected to real-time qPCR using Light Cycler (Roche Diagnostics, Mannheim, Germany). All primers and probes and amplification conditions for type I GnRH receptor and type II GnRH receptor were designed and described previously (Morimoto et al., 2005; Koga et al., 2000). Reaction parameters were as follows: For GnRHR, 40 cycles of denaturing ( $95^\circ\text{C}$ , 15 sec), annealing ( $62^\circ\text{C}$ , 10 sec), and extension ( $72^\circ\text{C}$ , 12 sec); for  $\beta$ -actin, 30 cycles at  $95^\circ\text{C}$  for 10 sec,  $60^\circ\text{C}$  for 10 sec, and  $72^\circ\text{C}$  for 10 sec. All PCR conditions were followed by melting curve analysis.

The gene expression levels of type I GnRH receptor and type II GnRH receptor were calculated and normalized by dividing the corresponding values of house keeping gene  $\beta$ -actin (GB accession number NM001101). Each PCR product was purified with a QIAEX II gel extraction kit (QIAGEN), and their identities were confirmed using an ABI

PRISM™ 310 genetic analyzer (Applied Biosystems, Foster city, CA).

**Antibodies used.** We performed immunohistochemical studies to investigate the immunoreaction of GnRHR in intact tissues. GnRHR (AT2.G7:sc-57176), a mouse monoclonal antibody against both type I and type II receptor (1:25 dilution), was used to immunolocalize GnRHR expression in tissues (Santa Cruz Biotechnology, Inc. CA). Ki-67 (MIB-1, Immunotech, Marseille, France), a mouse monoclonal antibody (1:100) was used to immunolocalize proliferating cells in biopsy specimens. Non-immune mouse immunoglobulin (Ig) G1 antibody in 1:50 dilution was used as a negative control.

**Immunohistochemistry.** The details of immunocytochemical or immunohistochemical staining were described elsewhere (Khan et al., 2003, 2004b, 2008; Ishimaru et al., 2004). We used at least two biopsies per patient and three slides per biopsy for immunohistochemical analysis.

The immunoreactivity of GnRHR in biopsy specimens was quantified by a modified method of quantitative-histogram score (Q-H score) as described recently (Khan et al., 2003, 2005c; Ishimaru et al., 2004). The Q-H score was calculated using the following equation:  $Q-H \text{ score} = \sum P_i (i+1)$ , where  $i = 1, 2 \text{ or } 3$  and  $P_i$  is the percentage of

stained cells for each intensity. The staining intensity was graded as 0 = no, 1 = weak, 2 = moderate, and 3 = strong. We calculated the mean Q-H scores of five different fields of one section by light microscopy at moderate magnification (x200). The cell proliferation index (Ki-67 index) in each tissue section was calculated by measuring the mean percentage of Ki-67-positive nuclei among total cells in four different microscopic fields (x200).

***Cell proliferation assays.*** 5-Bromo-2-deoxyuridine (BrdU) labeling and detection kit measures cell proliferation by quantitating BrdU incorporated into the newly synthesized DNA of replicating cells (Takagi, 1993). The incorporated BrdU can be detected by a quantitative cellular enzyme immunoassay (Biotrak, Amersham Pharmacia Biotech Ltd., UK) using monoclonal antibodies directed against BrdU. It offers a non-radioactive alternative to the [<sup>3</sup>H]-thymidine-based cell proliferation and carries equal sensitivity and specificity (Takagi, 1993). We examined the proliferation of epithelial cells, stromal cells and smooth muscle cells in response to variable doses ( $10^{-9}$ M to  $10^{-5}$ M) of GnRH agonist (Leuplin: leuprolide acetate, Takeda, Tokyo, Japan).

The detail procedure of BrdU incorporation assay was described previously

(Khan et al., 2005a, 2005b, 2005c). Briefly, desired cells (endometrial cells and smooth muscle cells) were cultured in 96 well microtitre plate ( $10^4$  cell/well). After a 24hr pre-incubation period without serum, respective cells were treated with or without GnRHa in a serum free medium and incubated for an additional 24 hours. After that, the cells were labeled with  $10 \mu\text{M}$  of BrdU ( $100 \mu\text{L/well}$ ) and incubated for 4 hours at  $37^\circ\text{C}$ . The cells were fixed and genomic DNA was denatured by adding  $200 \mu\text{L/well}$  of blocking reagent (1:10) for 30 minutes at room temperature (RT). Peroxidase-labeled anti-BrdU antibody (1:100) was added ( $100 \mu\text{L/well}$ ) and incubated for 90 minutes at RT. After washing three times, TMB (3,3',5,5'-tetramethylbenzidine) substrate solution was added ( $100 \mu\text{L/well}$ ) and incubated for 15 minutes at RT for color appearance and finally optical density was measured using a microplate reader at an absorbance of 450 nm. The intra-assay and inter-assay coefficients of variation were  $<10\%$  for this assay.

The values of BrdU incorporated cells in response to GnRH agonist were expressed as the percentage of controls (non-treated cells). The cells treated with BrdU diluent (PBS solution) was used as negative control. The absorbance values correlated directly to the amount of DNA synthesis and to the number of proliferating cells in

culture.

**Statistical analysis.** All results are expressed as either mean  $\pm$  SEM or median and inter-quartile range (IQR). The clinical characteristics of the subjects were compared with one-way analysis of variance and the  $X^2$  test for any difference between two groups. Mann-Whitney *U*-test or Student's *t*-test was used to analyze any difference in cell proliferations or protein expressions between two groups. For comparisons among groups, the Kruskal-Wallis test was used. A value of  $p < 0.05$  was considered to be statistically significant.

## Results

The clinical features of women with ovarian endometrioma, adenomyosis and uterine myoma between GnRHa-treated group and GnRHa-non-treated group and of women with pelvic endometriosis and control women are shown in Table 1. The duration of GnRHa therapy was also comparable among these three study groups, 4-6 months for women with ovarian endometrioma, 3-6 months each for women with adenomyosis and uterine myoma. Ten women with ovarian endometriosis, ten with adenomyosis, and eight with uterine myoma had coexisting peritoneal lesions.

*Gene expression of GnRH receptor in endometriosis, adenomyosis and uterine myoma.* As shown in Figure 1, standard RT-PCR analysis demonstrated amplified products of type I GnRH receptor and type II GnRH receptor in the eutopic endometria of control women and pelvic endometriosis as well as in cultured endometriotic epithelial cells and stromal cells (Figure 1, A). Both type I and type II GnRHR were also detected in tissues derived from cyst wall, adenomyotic tissue, myoma nodule and their corresponding eutopic endometria (Figure 1, B). Each PCR product was sequenced and confirmed to be identical to the sequence of type I GnRH receptor (Chi et



al., 1993) and type II GnRH receptor (Grundker et al., 2002). The relative gene levels of GnRHR in the eutopic and ectopic endometria are shown in Table 2. An almost similar level of GnRHR gene expression was observed in all studied cells and tissues.

***Immunoexpression of GnRHR based on menstrual cycle and r-ASRM***

***staging.*** The immunostaining of GnRHR was found to be weak during the proliferative phase (Figure 2A, upper column), moderate during the secretory phase (Figure 2A, middle column) and strong during the menstrual phase (Figure 2A, lower column). The immunoreactivity of GnRHR as measured by Q-H scores also showed the similar results (Figure 2B). The tissue localization of GnRHR was found in both gland cells and stromal cells. Comparing to control samples, GnRHR expression was found to be higher in samples derived from the secretory phase or menstrual phases of women with endometriosis (Figure 2B). We did not find any significant difference of GnRHR expression between samples derived from 35 women with stage I-II endometriosis and 25 women with stage III-IV endometriosis (Figure 2, A and B). We also found GnRHR expression in the endometria, pathologic lesions and myometria derived from women with endometrioma, adenomyosis and uterine myoma (data not shown).

***Immunoexpression of GnRHR in peritoneal lesions and adjacent peritoneum.***

When we distributed immunoexpression of GnRHR in different peritoneal lesions and their adjacent peritoneum of pelvic endometriosis, we found a significantly stronger immunoreaction and higher Q-H scores of GnRHR expression in the blood- filled opaque red lesions and their adjacent peritoneum ( $p < 0.05$  for both) when compared with that in non-opaque red lesions, black + white lesions and their adjacent peritoneum (Figure 3, A and B).

***Immunoexpression of GnRHR in GnRHa-treated and -non-treated samples.***

As shown in Figure 4, we did not find any significant difference in GnRHR immunoexpression and Q-H scores in the endometria (upper column), cyst wall (middle column) and coexisting peritoneal lesions (lower column) between GnRHa-treated and GnRHa-non-treated samples derived from women with ovarian endometrioma (Figure 4, A and B). We also found an insignificant difference in the expression of GnRHR in GnRHa-treated samples when compared with that of non-treated samples derived from women with adenomyosis and uterine myoma (data not shown).

***Effect of GnRHa on the proliferation of endometrial cells.*** After an initial

time-dependent study from day 1 to day 3, we found significant inhibitory response of GnRHa on cell proliferation from day 2 to day 3 without any variation among these days. Therefore, all the following dose-dependent studies were performed with an incubation period of 48 hours. We examined direct cell proliferation effect of a variable dose of GnRHa on epithelial cells and stromal cells derived from the endometria of 25 women with ovarian endometrioma, 20 women with adenomyosis and 36 women with uterine myoma. All these women had no GnRHa treatment before surgery.

As shown in Figure 5, GnRHa, at a concentration between  $10^{-9}$  and  $10^{-5}$  M, caused a significant and a dose-dependent inhibition of BrdU incorporation into DNA of endometrial epithelial cells and stromal cells derived from women with ovarian endometrioma (A), adenomyosis (B) and uterine myoma (C). The maximal anti-proliferative effect (30-32% decrease below the control) was observed at  $10^{-6}$  M and  $10^{-5}$  M concentration of leuprolide acetate.

Although GnRHa was able to inhibit BrdU incorporation from a lower ( $10^{-9}$  M and  $10^{-8}$  M) to higher doses ( $10^{-7}$  M to  $10^{-5}$  M) into endometrial cells derived from women with endometrioma and uterine myoma (Figure 5, A and C), GnRHa was able to

inhibit BrdU incorporation in response to only higher doses ( $10^{-6}$  M and  $10^{-5}$  M) into endometrial cells derived from women with adenomyosis (Figure 5, B). We could exclude the cytotoxic effect of GnRHa on endometrial cells by trypan blue exclusion test. In all experiments, spontaneously detached and dead cells were not different between GnRHa-non-treated and GnRHa-treated cells (data not shown).

***Effect of GnRHa on the proliferation of cells derived from pathologic lesions of women with endometrioma, adenomyosis and uterine myoma.*** We examined direct cell proliferation effect of GnRHa on cyst wall stromal cells derived from women with ovarian endometrioma and on smooth muscle cells derived from each of adenomyotic lesions and myoma nodules (Figure 6).

We found that GnRHa (leuprolide acetate) was able to significantly suppress BrdU incorporation into cyst wall stromal cells at a dose of  $10^{-7}$  M to  $10^{-5}$  M (Figure 6A,  $p < 0.05$  vs. non-treated cells), into smooth muscle cells derived from adenomyotic lesions at a dose of  $10^{-6}$  M and  $10^{-5}$  M (Figure 6B,  $p < 0.05$  vs. non-treated cells), and into smooth muscle cells derived from myoma nodules at a dose of  $10^{-8}$  M to  $10^{-5}$  M (Figure 6C,  $p < 0.05$  vs. non-treated cells).

We did not find any significant anti-proliferative effect of GnRHa (leuprolide acetate) on cells derived from both endometria (data not shown) and pathologic lesions in 8/25 (32%) of women with endometrioma, in 6/20 (30%) of women with adenomyosis, and in 10/36 (28%) of women with uterine myoma (Figure 7).

***Effect of GnRHa on the proliferation of cells derived from the eutopic and ectopic endometria of women with pelvic endometriosis.*** In addition to its effect on cells derived from the endometria and pathologic lesions of women with ovarian endometrioma, adenomyosis and uterine myoma, we also found that GnRHa retains its direct cell proliferation effect on cells derived from women with pelvic endometriosis.

As shown in Figure 8, GnRHa was able to significantly suppress BrdU incorporation into epithelial cells and stromal cells derived from the eutopic endometria (A) and ectopic endometria (B) of women with pelvic endometriosis. The direct anti-proliferative effect of GnRHa on eutopic and ectopic endometrial cells was observed from a lower dose to high dose ( $10^{-8}$  M to  $10^{-5}$  M) (Figure 8, A and B). We did not find any significant anti-proliferative effect of GnRHa on cells derived from 8/35 (23%) of women with pelvic endometriosis (data not shown).

***Immunoexpression of Ki-67 in endometria and pathologic lesions.*** In order to examine the cell proliferation effect of GnRHa in intact tissue, we investigated immunoreaction of Ki-67, a cell proliferation marker, in GnRHa-treated and -non-treated biopsy specimens derived from the endometria and respective pathologic lesions of women with ovarian endometrioma, adenomyosis and uterine myoma.

Figure 9 shows the tissue localization of Ki-67 in the endometria and pathologic lesions of GnRHa-treated and -non-treated biopsy specimens derived from women with ovarian endometrioma (A), adenomyosis (B) and uterine myoma (C). We found that Ki-67 index was significantly lower in both endometria (Figure 10, A) and pathologic lesions (Figure 10, B) of GnRHa-treated samples derived from women with endometrioma, adenomyosis and uterine myoma when compared with the corresponding Ki-67 index in GnRHa-non-treated samples derived from these three groups of women. Although data not shown, we also found a similarly decreased pattern of Ki-67 index in the GnRHa-treated myometria derived from women with adenomyosis and uterine myoma.

## **Discussion**

Although a few previous literatures demonstrated the anti-proliferative effect of exogenous GnRH agonist or endogenous GnRH II on endometrial cells derived from women with endometriosis (Borroni et al., 2000; Morimoto et al., 2005), we further demonstrated the direct anti-proliferative effect of GnRHa on the cells derived from endometria and pathologic lesions of four groups of women such as women with ovarian endometriosis, pelvic endometriosis, adenomyosis and uterine myoma. Based on the limited information on the presence of GnRHR expression in peripheral tissues, we could detect both gene and protein expression of GnRHR in cells and tissues derived from these women.

We found that exogenous treatment with a variable concentration of GnRHa was able to significantly suppress the proliferation of cells derived from the endometria and pathologic lesions of women not only with endometriosis but also of women with adenomyosis and uterine myoma. These direct anti-proliferative effects of GnRHa in vitro correspond to in vivo results of Ki-67, a cell proliferation marker, in intact tissues. In fact, Ki-67 index was significantly lower in both endometria and pathologic lesions

derived from GnRHa-treated women than from GnRHa-non-treated women. Although we could not exclude the hypo-estrogenic effect of GnRHa on the changes in Ki-67 index, our in vitro cell proliferation study confirmed direct anti-proliferative effect of GnRHa on pathologic lesions.

Additional interesting findings of our current study are that both eutopic and ectopic endometrial cells derived from women with pelvic endometriosis equally express GnRHR in both gene and protein level and GnRHa also retains its direct anti-proliferation effect on both eutopic and ectopic endometrial cells derived from women with pelvic endometriosis. This indicates that when women with ovarian endometrioma, adenomyoma and uterine myoma are treated with GnRHa, this estrogen suppressing agent is equally effective in reducing the growth of peritoneal lesions coexisting with these reproductive diseases.

Although we did not find any significant difference in GnRHR protein expression between early (stage I-II) and advanced endometriosis (stage III-IV), we found an increased transition of GnRHR expression among different phases of the menstrual cycle. Our results are in consistent with the previous results that demonstrated



protein expression of GnRHR throughout all phases of menstrual cycle with a significant increase in the secretory phase when compared with the proliferative phase (Casañ et al., 1998; Raga et al., 1998). Again, we did not find any significant difference in GnRHR protein expression in biopsy samples between GnRHa-treated and GnRHa-non-treated women with endometrioma, adenomyosis and uterine myoma. This indicates that unlike central pituitary action, the anti-proliferative effect of GnRHa on each cell type may not depend on the down-regulation of GnRHR rather on the amount and binding affinity of GnRHa for its receptor in peripheral tissues. Further studies are needed to clarify our current findings.

We also found higher immunoexpression of GnRHR in the blood-filled opaque red lesions and their adjacent peritoneum than in non-opaque or other less active peritoneal lesions and their adjacent peritoneum derived from women with pelvic endometriosis. This could be due to increased production of different pro-inflammatory cytokines and growth factors by opaque red lesions than by other peritoneal lesions (Khan et al., 2004a). These results are biologically significant. GnRHa treatment may not only suppress the growth of peritoneal lesions coexisting with these reproductive diseases, this

treatment may also reduce blood loss or inflammatory reaction in pelvic environment. In fact, we recently demonstrated that GnRHa was able to suppress inflammatory response, angiogenic response and induce apoptosis in women with endometriosis, adenomyosis and uterine myoma (Khan et al., 2010).

One important finding of our current study is that exogenous treatment with similar doses of GnRHa did not affect cell growth in 23 % of women with pelvic endometriosis, 32% of women with endometrioma, 30% of women with adenomyosis, and 28% of women with uterine myoma regardless of the expression of GnRHR. Our results are supported by the study of Borroni et al. (2000) who also found no anti-proliferative effect of GnRHa (leuprolide acetate) on endometriotic cells derived from 60% of cases with ovarian endometriosis. In addition, we found a differential cellular response of GnRHa in different diseases, more in cells from endometriosis and myoma and less in cells from adenomyosis. This cellular variation in growth inhibition in response to GnRHa treatment can be explained by a difference in cellular histogenesis or a difference in GnRH receptor-ligand binding affinity in cells derived from women with different reproductive diseases. In fact, previous studies investigating the presence of

GnRH receptor in different tissues revealed the presence of high affinity/low capacity and low affinity/high capacity binding sites (Qayum et al., 1990; Limonta et al., 1992; Emons et al., 1993) that may coexist in the same tissue.

Leuprolide acetate, the type of GnRHa, as we used in our current study, has been found to interact with type I GnRH receptor, where type II GnRH receptor functions as a co-receptor (Borroni et al., 2000; Imai et al., 2000). In our present study, concentrations of GnRHa used are within the pharmacological range in the peripheral circulation. In human, serum leuprolide acetate concentration after subcutaneous injection of 1.88 mg and 3.75 mg may range from  $10^{-8}$  M to  $10^{-7}$  M and  $0.5 \times 10^{-6}$  M to  $10^{-5}$  M, respectively, over a five-week period (Mazzei et al., 1990). Thus, theoretically, this compound may affect endometrial cell proliferation also in vivo.

Finally we conclude that in addition to ovarian endometriosis, peripheral tissues derived from women with pelvic endometriosis, adenomyosis, and uterine myoma equally express GnRHR at the gene and protein level. In addition to hypo-estrogenic effect and other multifunctional roles of GnRHa in peripheral tissues as we described recently (Khan et al., 2010), GnRHa may also exert direct anti-proliferative effect on

different cells derived from women with these reproductive diseases. All these biological functions of GnRHa may be involved in the regression of these diseases with consequent remission of symptoms. Further studies are needed to strengthen our current findings.

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## **Figure legends**

**Figure 1.** Standard RT-PCR analysis of the expression of type I GnRH receptor and type II GnRH receptor mRNA in the eutopic endometrial tissues of control woman (lane 1), woman with pelvic endometriosis (lane 2), isolated epithelial cells (lane 3) and stromal cells (lane 4) are shown in the upper panel (**A**). The lower panel (**B**) shows the mRNA expression of type I and type II GnRH receptor in the eutopic endometrium (lane 1) and cyst wall (lane 2) of woman with endometrioma, in the eutopic endometrium (lane 3) and adenomyotic tissues (lane 4) of woman with adenomyosis, in the eutopic endometrium (lane 5) and nodule (lane 6) of woman with uterine myoma. The PCR products show the predicted sizes on the basis of cDNA sequence. M represents molecular marker; RT (-) indicates negative control with no reverse transcription. PCR band of each lane is the representative of six different samples with similar results.

**Figure 2.** Shows immunohistochemical staining of GnRHR (**A**) and quantitative-histogram (Q-H) scores of GnRHR immunoreactivity (**B**) in eutopic endometrial tissues derived from 30 control women, 35 women with pelvic endometriosis

and 25 women with ovarian endometriosis based on phases of menstrual cycle and r-ASRM staging of endometriosis. Kruskal-Wallis test indicated that Q-H scores of GnRHR were the highest in the menstrual phase, intermediate in the secretory phase and lowest in the proliferative phase (**B**). No significant difference was observed in the Q-H scores of GnRHR expression between stage I-II endometriosis (hatched bar) and stage III-IV endometriosis (black bar) (**B**). Boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values.

**Figure 3.** Shows immunohistochemical staining of GnRHR (**A**) and quantitative-histogram (Q-H) scores of GnRHR immunoreactivity (**B**) in different peritoneal lesions (white bar) based on color appearance and their adjacent peritoneum (black bar) derived from 35 women with pelvic endometriosis. The left column of upper Figure (A) shows H-E staining of respective peritoneal lesions, middle column shows immunostaining of GnRHR in peritoneal lesions, and right column shows immunostaining of GnRHR in adjacent peritoneum of respective peritoneal lesions. The

Q-H scores of GnRHR expression were significantly higher in the blood-filled opaque red lesions and their adjacent peritoneum (\* $p < 0.05$  for each) when compared with that in non-opaque red lesions or black + white lesions. Boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values.

**Figure 4.** Shows immunohistochemical staining of GnRHR (A) and quantitative-histogram (Q-H) scores of GnRHR immunoreactivity (B) in GnRHa-treated (right column, A) and -non-treated (left column, A) endometria (upper column), cyst wall (middle column) and coexisting peritoneal lesions (lower column) derived from women with ovarian endometrioma. Nonimmune mouse IgG-stained slides are shown against each specimen. We did not find any significant difference in the Q-H scores of GnRHR expression in samples between GnRHa-non-treated (n=25) (white bar) and GnRHa-treated (n=20) (black bar) women with endometrioma (B). Boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values.

**Figure 5.** Shows effects of GnRHa (leuprorelin acetate) on BrdU incorporation into epithelial cells (black bar) and stromal cells (open bar) derived from the eutopic endometria of women with ovarian endometrioma (**A**), adenomyosis (**B**) and uterine myoma (**C**). Both GnRHa-treated and -non-treated endometrial epithelial cells and stromal cells were incubated for 48hr. Results are shown as the mean percentage of the untreated cells ( $\pm$  SEM) of triplicate experiments using cells derived from different women who were responsive to GnRHa. Back and white bar at far right indicate negative control of cells treated with BrdU diluent. \* $p < 0.05$  vs. non-treated cells for **A**, **B**, and **C**.

**Figure 6.** Shows effects of GnRHa (leuprorelin acetate) on BrdU incorporation into stromal cells (**A**) derived from cyst wall of women with ovarian endometrioma and smooth muscle cells derived from the pathologic lesions of women with adenomyosis (**B**) and uterine myoma (**C**). Cyst wall stromal cells and respective smooth muscle cells were treated with GnRHa at the indicated concentration for 48hr. Results are shown as the mean percentage of the untreated cells ( $\pm$  SEM) of triplicate experiments using cells derived from different women who were responsive to GnRHa. White bar indicates

negative control of cells treated with BrdU diluent. \* $p < 0.05$  vs. non-treated cells for **A**, **B**, and **C**.

**Figure 7.** Shows effects of GnRHa (leuprorelin acetate) on BrdU incorporation into stromal cells (**A**) derived from cyst wall of eight women with ovarian endometrioma and smooth muscle cells derived from the pathologic lesions of six women with adenomyosis (**B**) and 10 women with uterine myoma (**C**) who were resistant to the effect of GnRHa.

We did not find any difference in BrdU incorporation into each of these cells at the indicated dose of GnRHa. Results are shown as the mean percentage of the untreated cells ( $\pm$  SEM) of triplicate experiments using cells derived from these three groups of women.

White bar indicates negative control of cells treated with BrdU diluent.

**Figure 8.** Shows effects of GnRHa (leuprorelin acetate) on BrdU incorporation into epithelial cells (black bar) and stromal cells (open bar) derived from the eutopic endometria (**A**) and ectopic endometria (**B**) of 35 women with pelvic endometriosis. Both GnRHa-treated and non-treated epithelial cells and stromal cells were incubated for 48hr.

Results are shown as the mean percentage of the untreated cells ( $\pm$  SEM) of triplicate experiments using cells derived from women who were responsive to GnRHa.. \* $p < 0.05$  vs. non-treated cells for **A** and **B**.

**Figure 9.** Shows immunohistochemical staining of Ki-67, a cell proliferation marker, in the GnRHa-non-treated (upper column, **A**, **B** and **C**) and GnRHa-treated (lower column, **A**, **B** and **C**) endometria and pathologic lesions derived from women with ovarian endometrioma (upper left block, **A**), adenomyosis (upper right block, **B**) and uterine myoma (lower block, **C**). GnRHa (-), GnRHa-non-treated samples; GnRHa (+), GnRHa-treated samples.

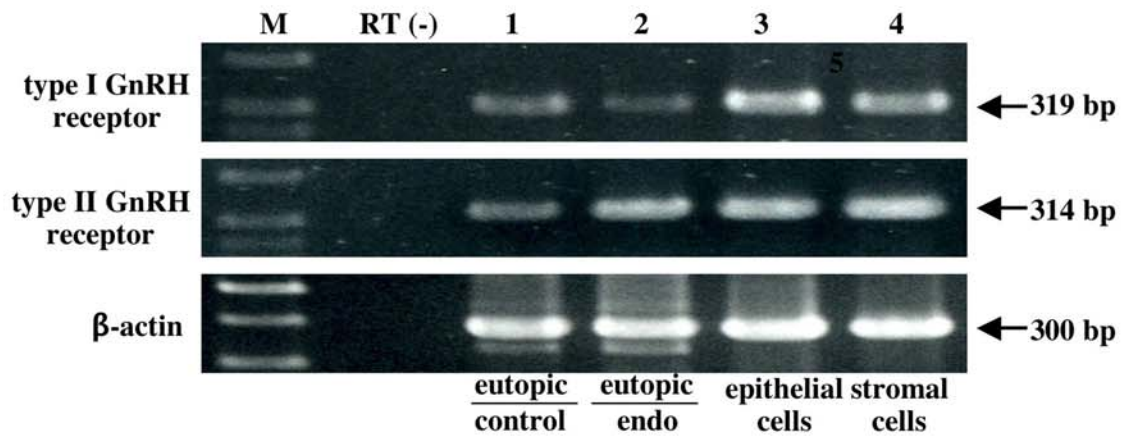
**Figure 10.** Shows Ki-67 index (percentage of Ki-67 immunoreactive cells among total cells) in the biopsy specimens derived from the endometria (**A**) and pathologic lesions (**B**) of GnRHa-non-treated (white box) and GnRHa-treated (hatched box) women with ovarian endometrioma, adenomyosis and uterine myoma. The Ki-67 indices were found to be significantly lower in the GnRHa-treated samples when compared with that in



GnRHa-non-treated samples ( $p < 0.05$  for each). Boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values.

Figure 1.

**A**



**B**

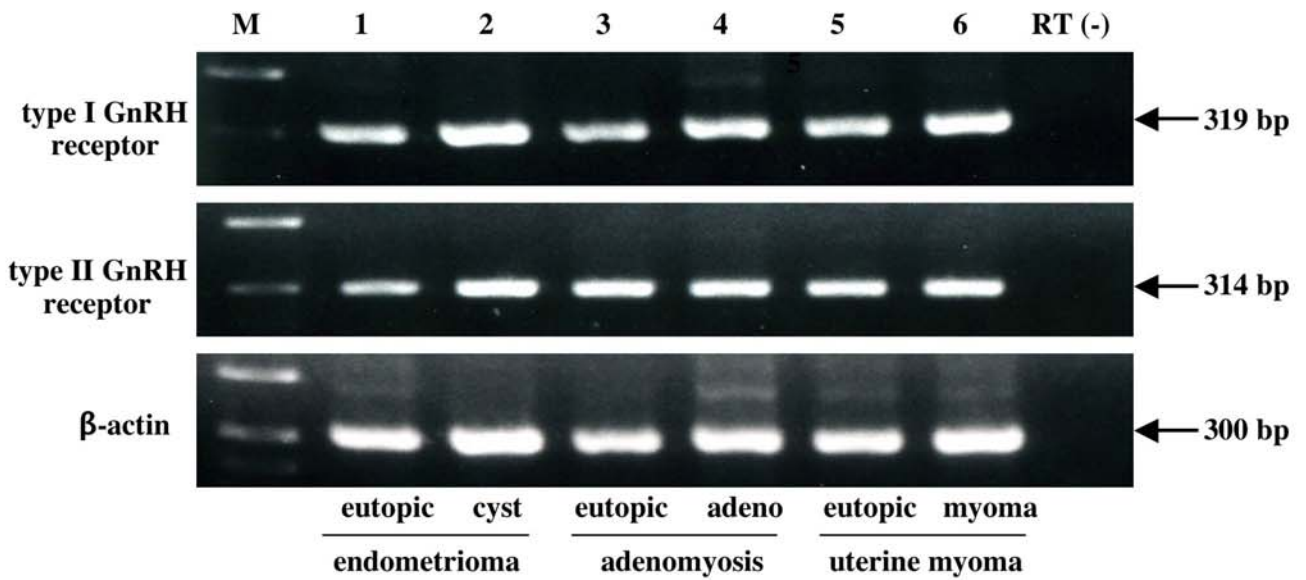


Figure 2

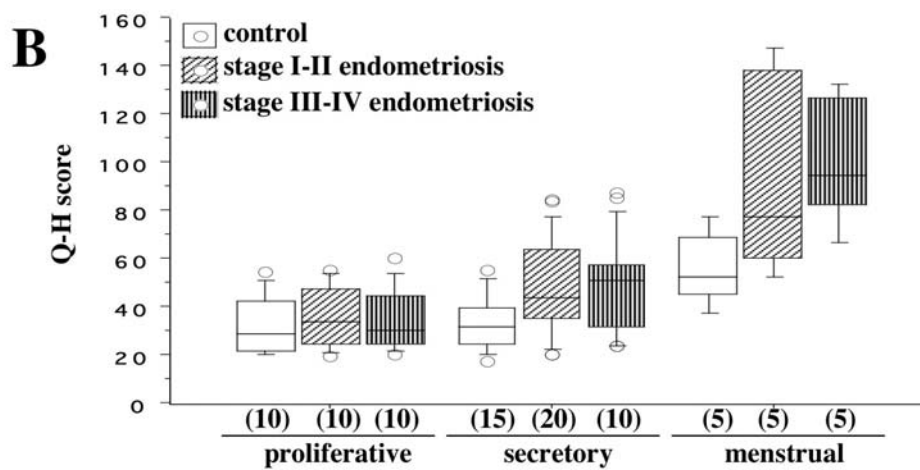
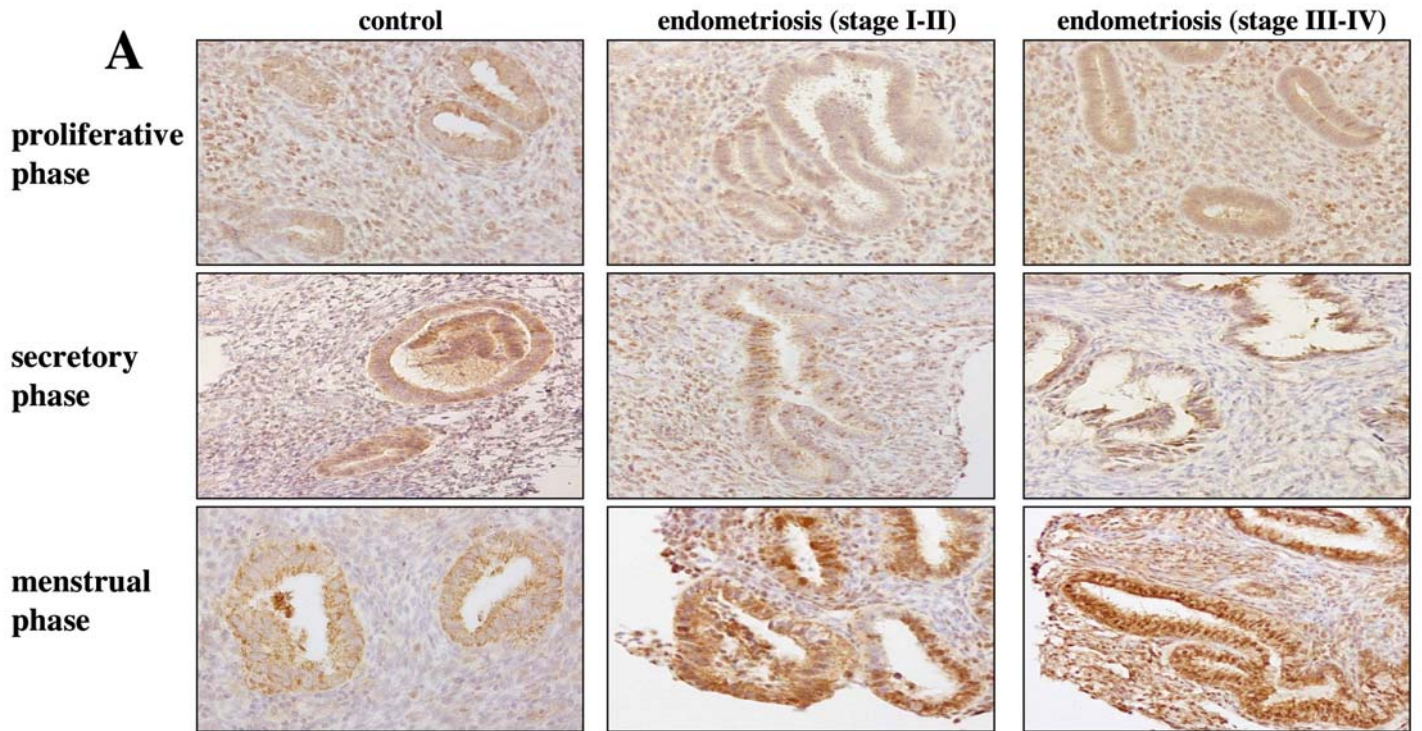




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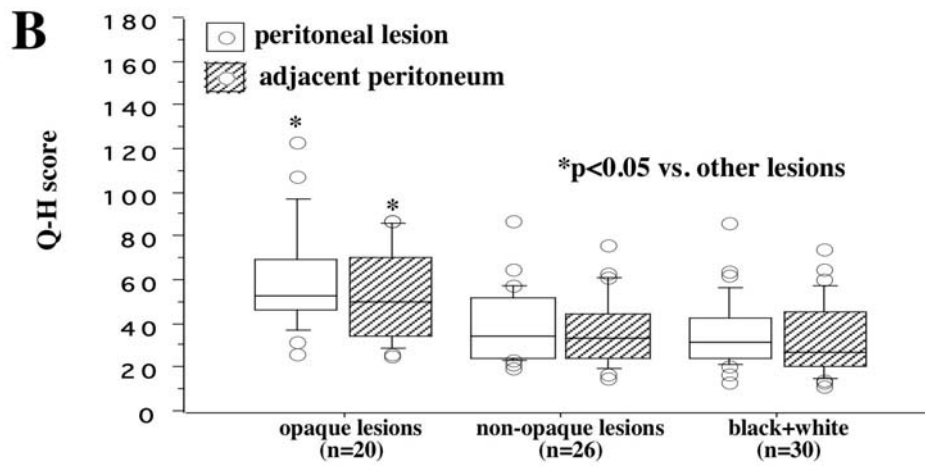
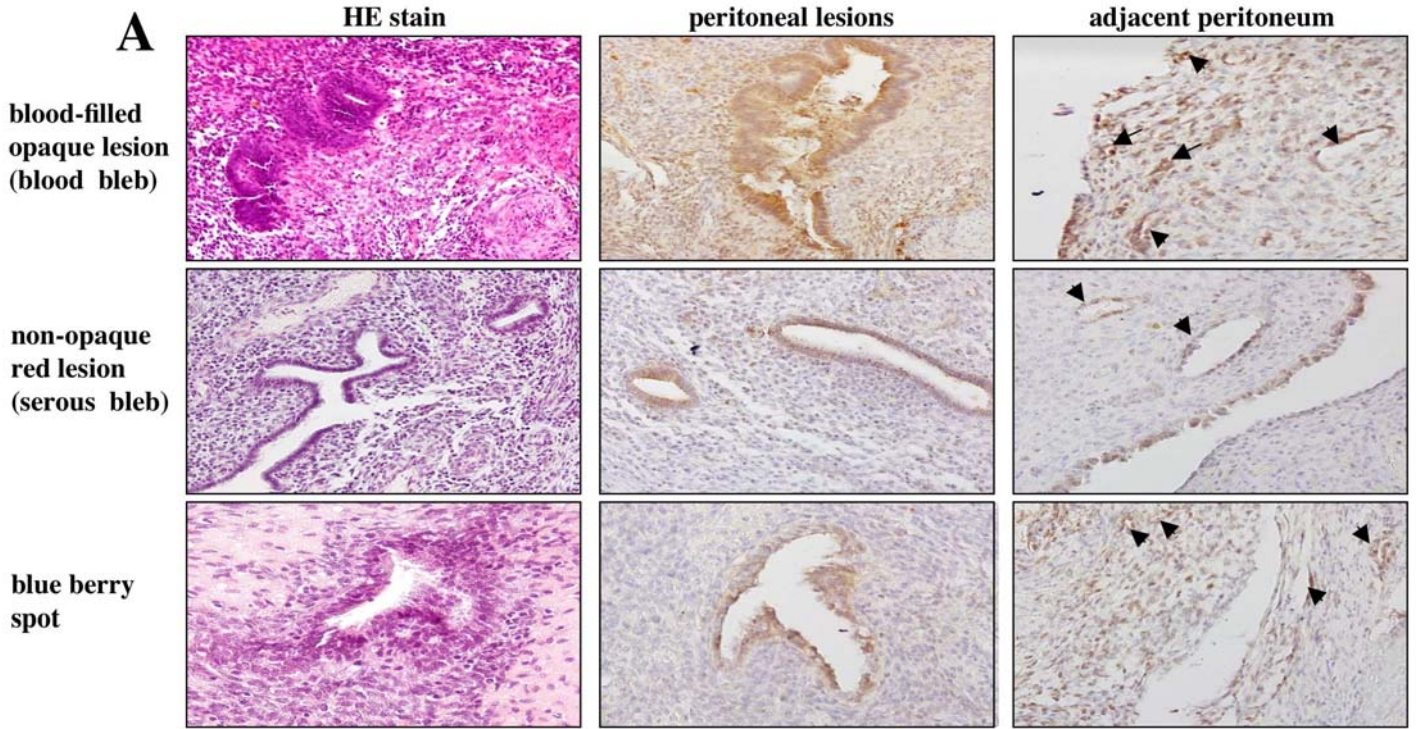


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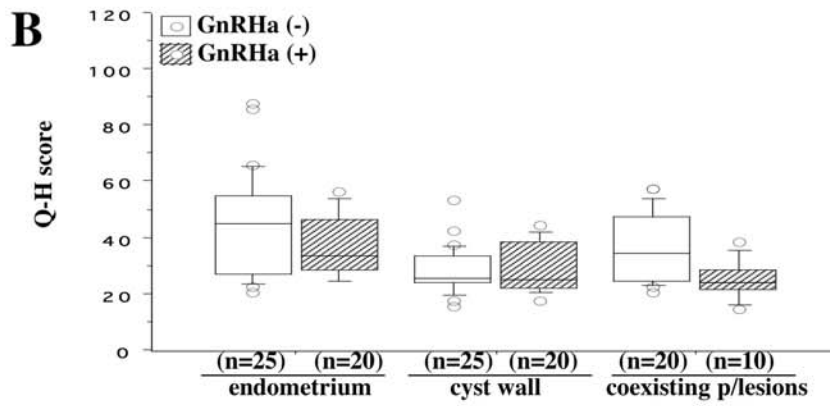
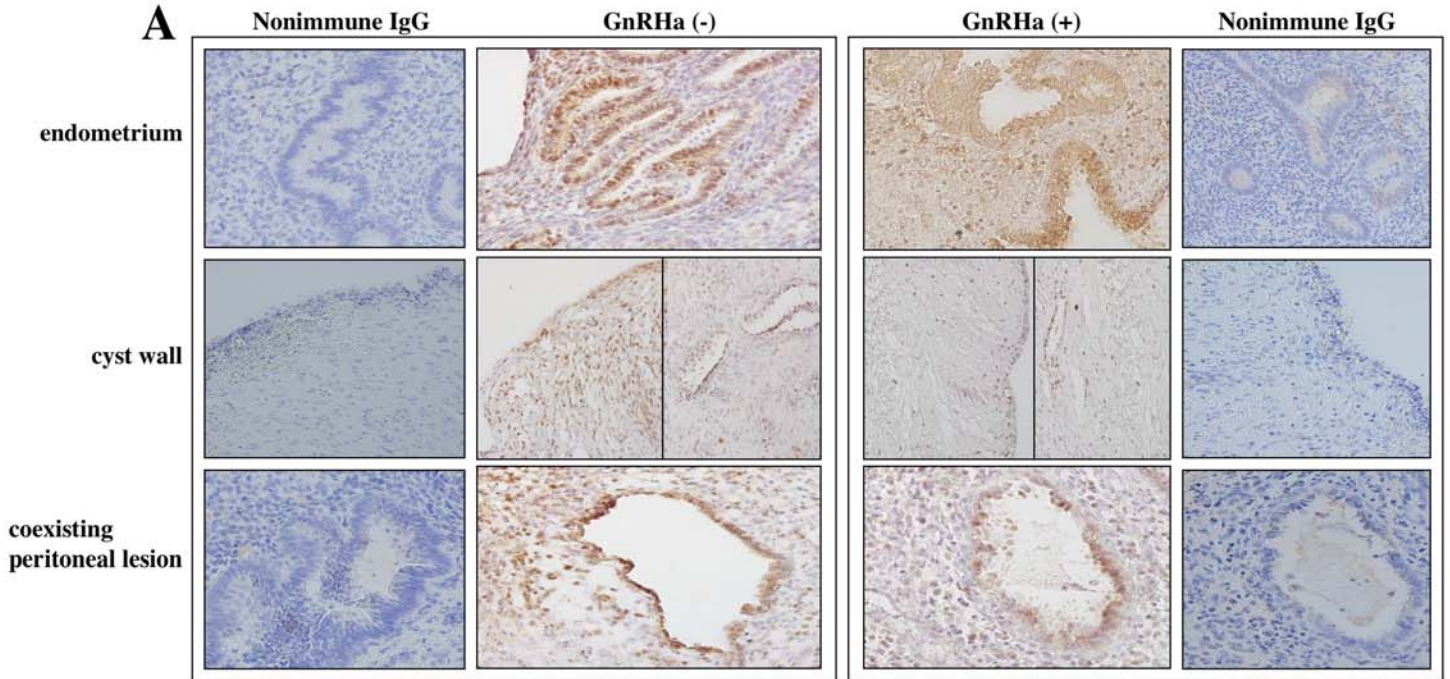


Figure 5

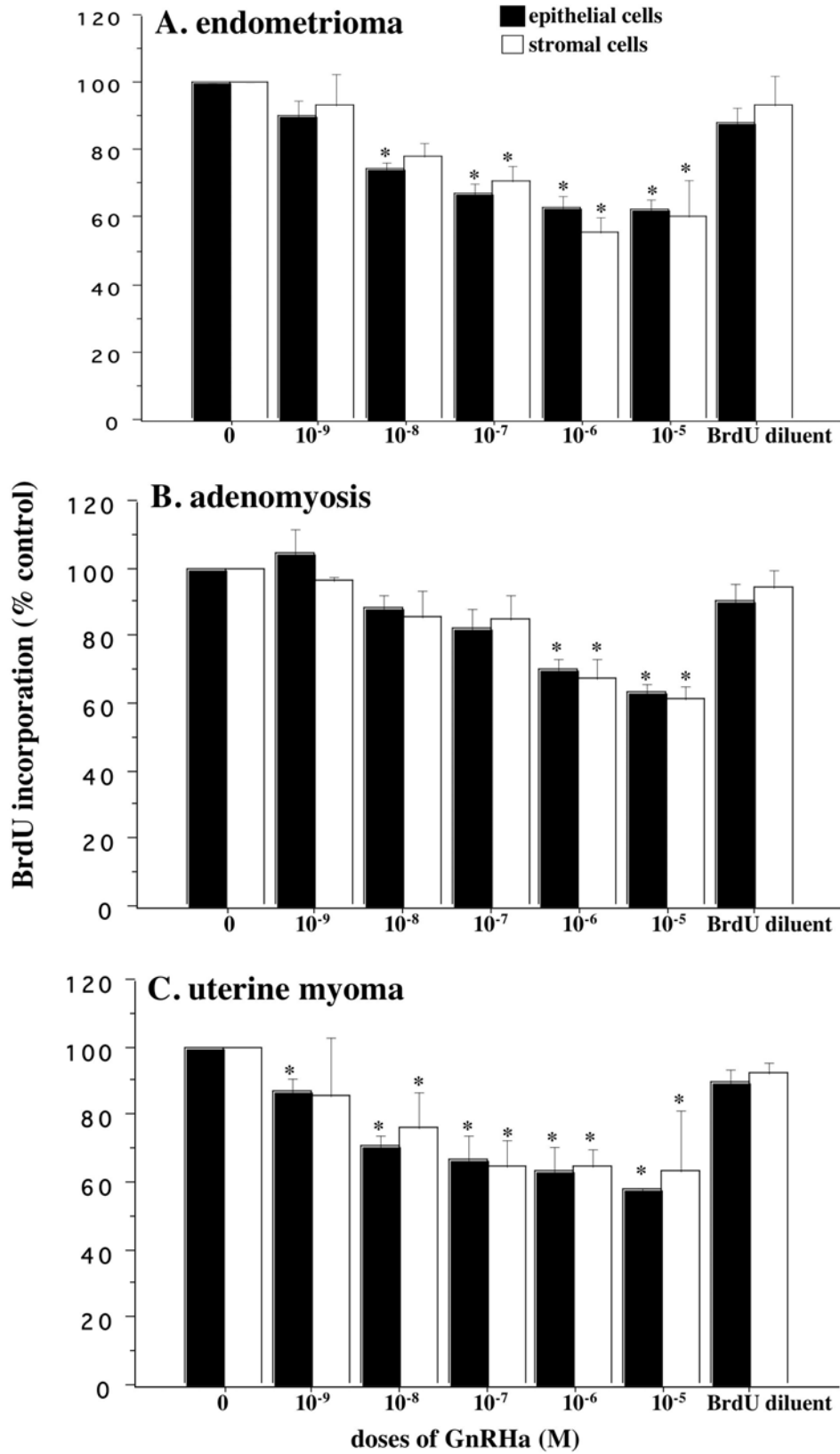


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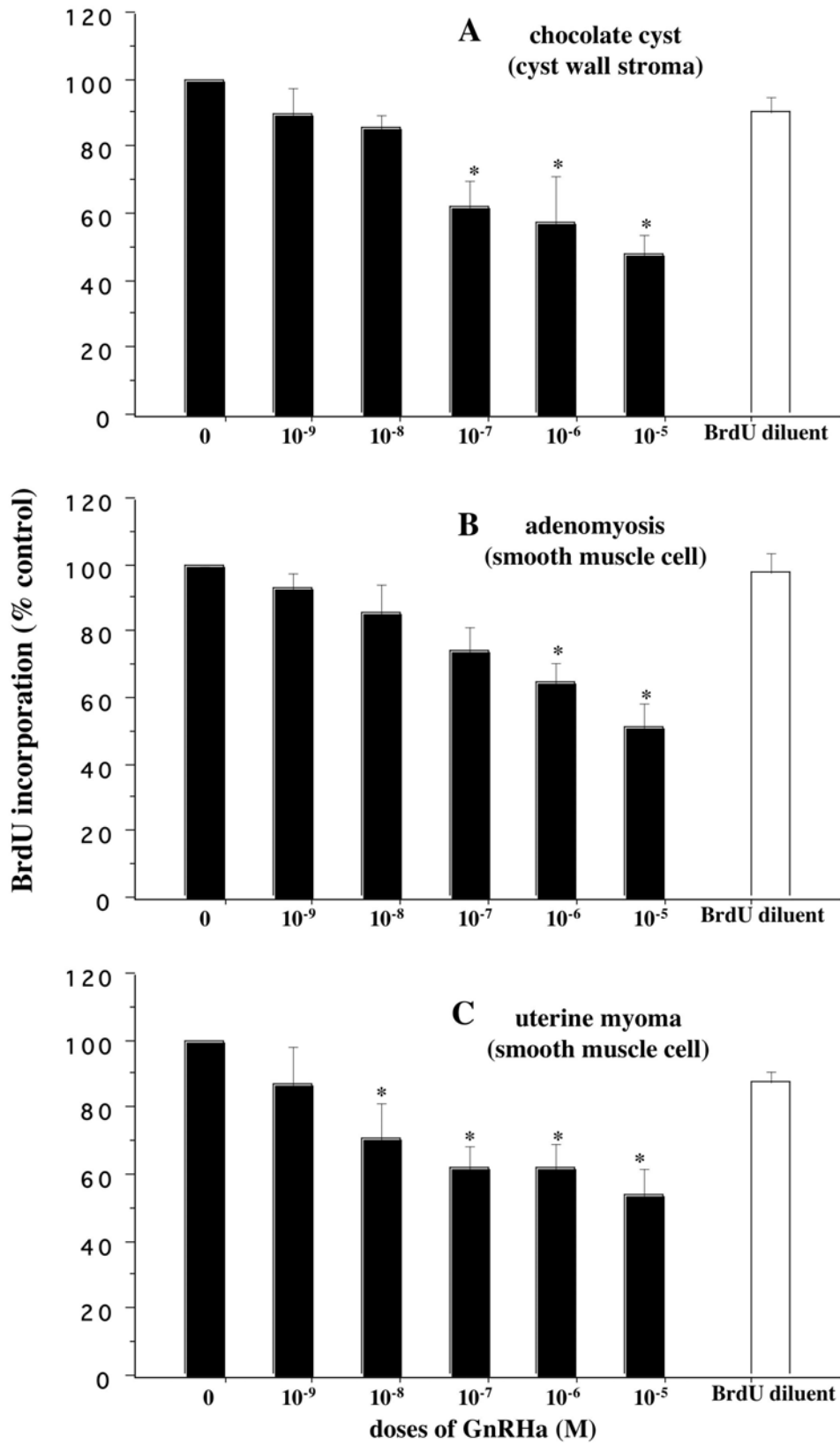


Figure 7

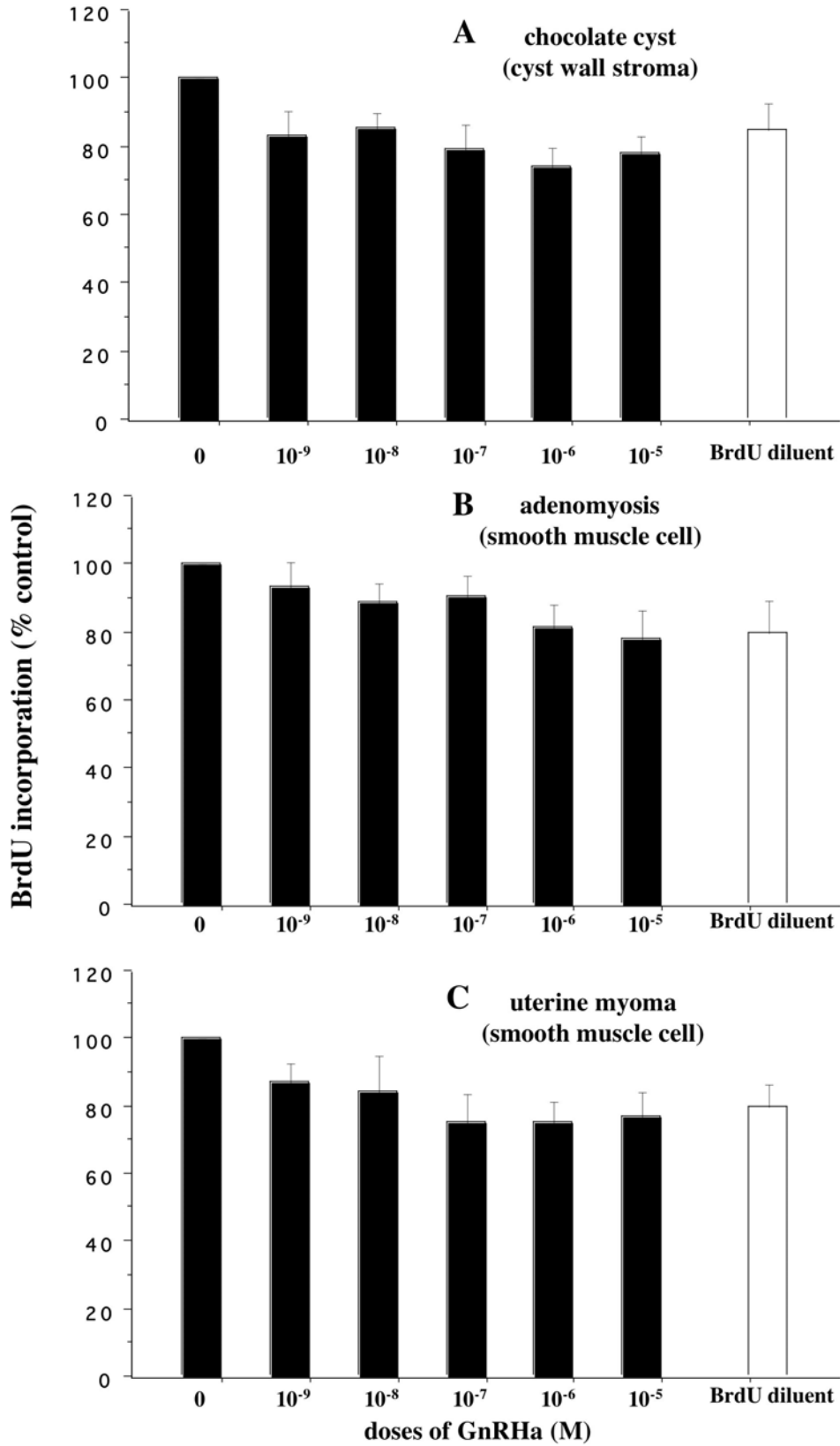




Figure 8

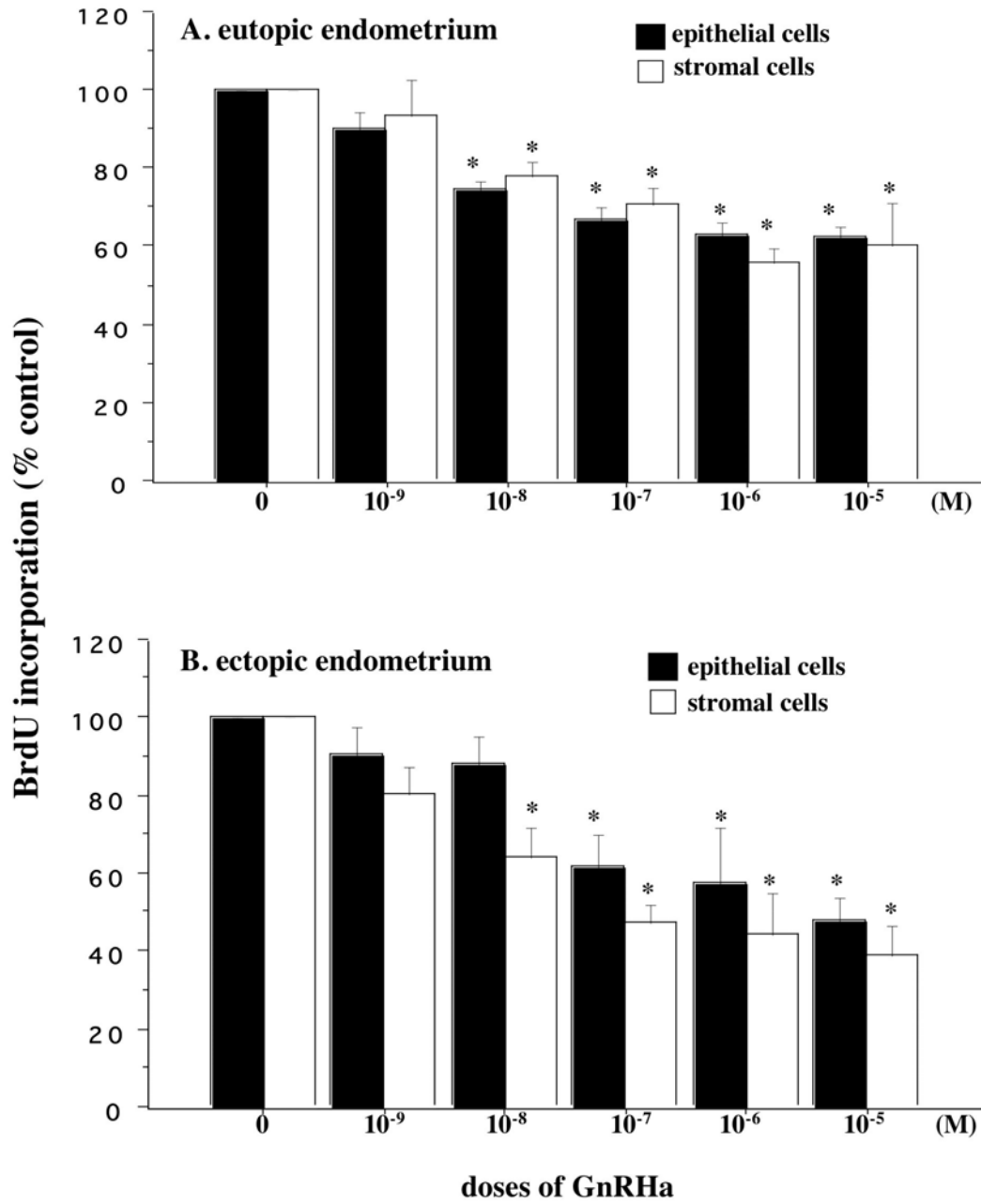


Figure 9

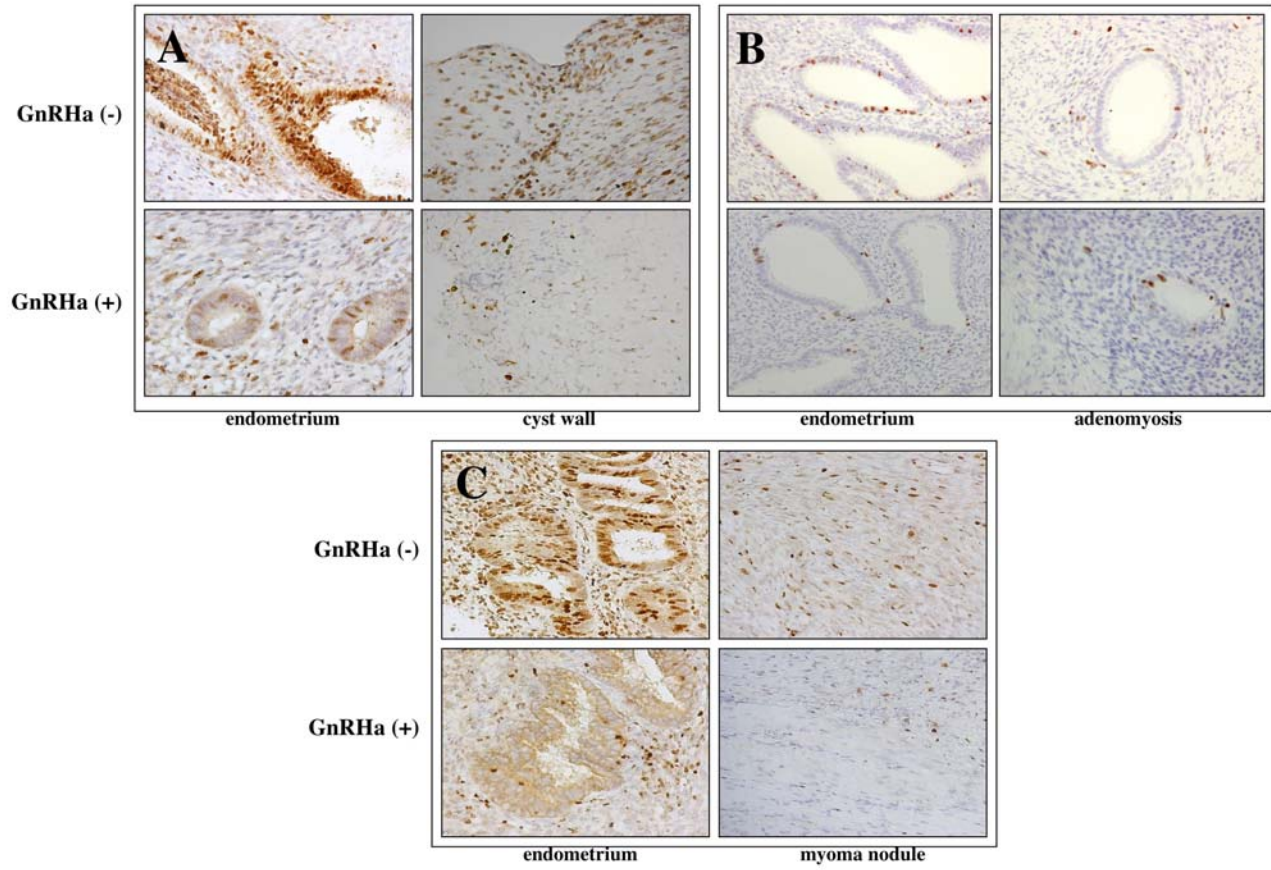
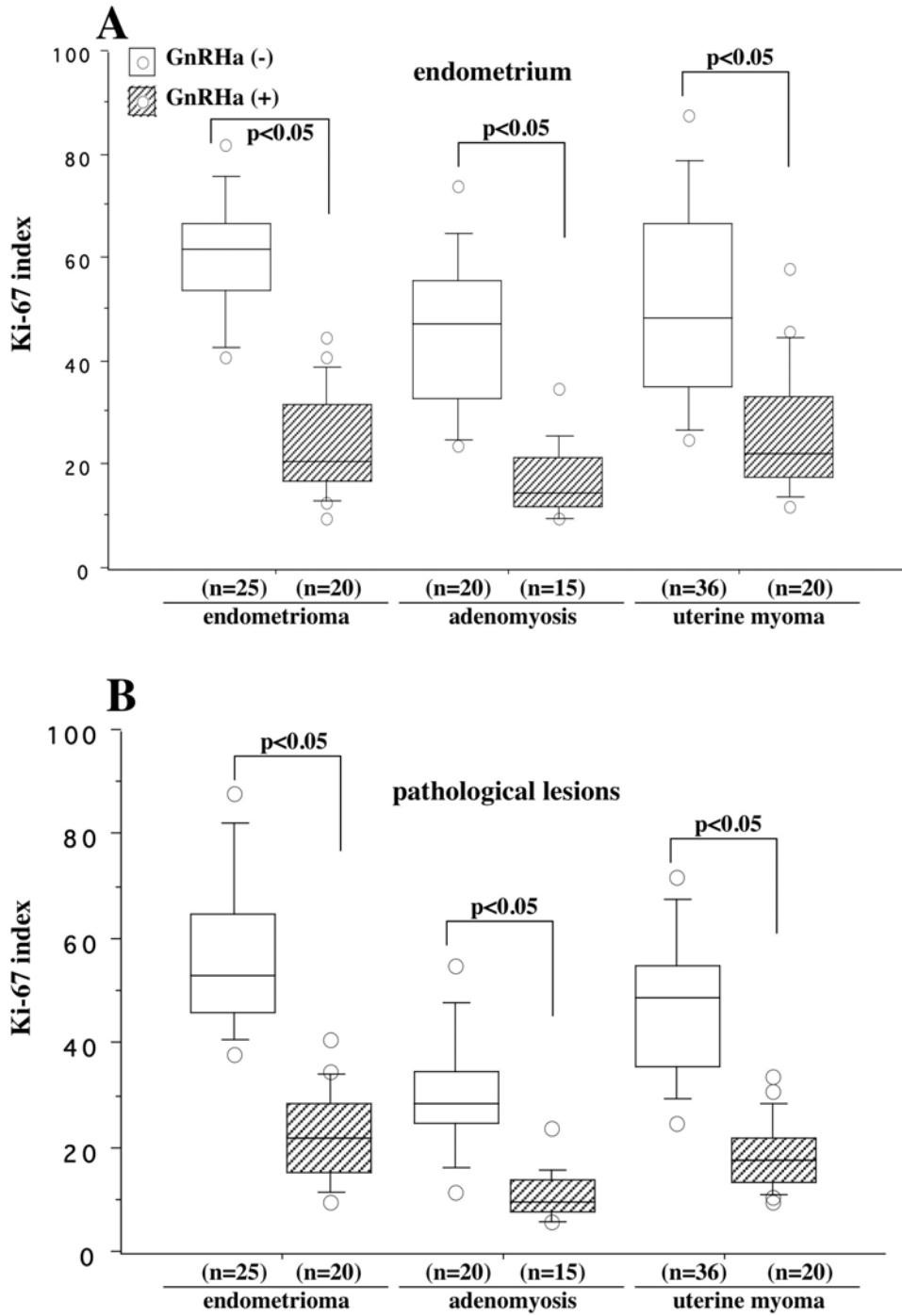


Figure 10



**Table 1. Clinical profiles of women in the study group and control group.**

	GnRHa (-)	GnRHa (+)
<b>Ovarian endometrioma (n=45)</b>	25	20
age in yrs (mean $\pm$ SD)	30.4 $\pm$ 3.7	31.5 $\pm$ 2.4
size in cm (mean $\pm$ SD)	6.0 $\pm$ 2.1	7.5 $\pm$ 1.4
menstrual cycle: P/S/M/A	8/15/2/0	0/0/0/20
duration of therapy (month)		4-6
r-ASRM stage III-IV	25	20
<b>Adenomyosis (n=35)</b>	20	15
age in yrs (mean $\pm$ SD)	41.9 $\pm$ 4.0	42.1 $\pm$ 2.6
menstrual cycle: P/S/M/A	8/12/0/0	0/0/0/15
duration of therapy (month)		3-6
hysterectomy done (n=25)	15	10
<b>Uterine myoma (n=56)</b>	36	20
age in yrs (mean $\pm$ SD)	39.1 $\pm$ 6.2	36.1 $\pm$ 5.8
size in cm (mean $\pm$ SD)	5.0 $\pm$ 2.1	6.1 $\pm$ 1.3
menstrual cycle: P/S/M/A	12/18/6/0	0/0/0/20
duration of therapy (month)		3-6
hysterectomy done (n=27)	15	12
<b>Pelvic endometriosis (n=35)</b>	35	
age in yrs (mean $\pm$ SD)	28.6 $\pm$ 4.2	
menstrual cycle: P/S/M	10/20/5	
r-ASRM stage I-II	35	
opaque/non-opaque/black+white	20/26/30	
<b>Control group (n=30)</b>	30	
age in yrs (mean $\pm$ SD)	28.4 $\pm$ 3.9	
menstrual cycle: P/S/M	10/15/5	
dermoid/serous or mucinous cyst	22/8	

The results are expressed as mean  $\pm$  SD. GnRHa (-), without GnRH agonist therapy; GnRHa (+), with GnRH agonist therapy; P, proliferative phase; S, secretory phase; M, menstrual phase; A, amenorrhea; r-ASRM, revised staging of American Society of Reproductive Medicine.

**Table 2. Relative gene levels of gonadotropin releasing hormone receptor (GnRHR) in the eutopic and ectopic endometrium.**

	control eutopic	pelvic endo eutopic	pelvic endo ectopic		endometrioma		adenomyosis		uterine myoma	
			epithelial	stroma	eutopic	cyst wall	eutopic	lesion	eutopic	nodule
Type I GnRHR	0.78 ± 0.16	0.82 ± 0.21	1.12 ± 0.15	1.08 ± 0.12	1.15 ± 0.17	1.21 ± 0.15	1.20 ± 0.12	1.18 ± 0.12	1.21 ± 0.14	1.20 ± 0.15
Type II GnRHR	0.89 ± 0.15	0.96 ± 0.18	1.18 ± 0.17	1.15 ± 0.11	1.12 ± 0.12	1.18 ± 0.21	1.22 ± 0.15	1.20 ± 0.17	1.22 ± 0.15	1.18 ± 0.12

Non-treated eutopic and ectopic endometrial cells and tissues were derived from control women and women with pelvic endometriosis, ovarian endometrioma, adenomyosis and uterine myoma. The expression of type I and type II GnRHR mRNA was determined using real-time quantitative polymerase chain reaction and was expressed as fold changes in relative gene levels compared with housekeeping  $\beta$ -actin gene. All values were expressed as mean  $\pm$  SEM of three independent experiments. An almost similar basal levels of type I and type II GnRHR gene expression was observed in either eutopic or ectopic endometria of women with these reproductive diseases.