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The Expression of Hepatoma Upregulated Protein in Human Endometrium during the Menstrual Cycle

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Abstract

Aims

Human endometrium resists embryo implantation except during the window period. Currently, uterine HURP expression is known to be involved in endometrial stromal proliferation during embryo implantation of mice. Thus, we demonstrated hepatoma up-regulated protein (HURP) expression in the human endometrium during the menstrual cycle, as well as HURP regulation in endometrial stromal cells (ESCs).

Materials and Methods

We collected human endometrial samples from different menstrual cycle phases (early/late proliferative, and early/mid/late secretory), and then analyzed these samples by immunohistochemistry, reverse transcription-polymerase chain reaction, and Western blotting. We also assessed the effects of two sex-steroid hormones, 17β-estradiol (E2) and 4-pregnene-3,20-dione (P4) on the cultured stromal cells.

Results

HURP protein was localized to the nucleus of the endometrial both epithelial and stromal cells in all stages. Also, HURP mRNA and protein in human endometrial tissue was significantly up-regulated during late-proliferative and secretory phase, compared with early-proliferative phase. In ESCs, HURP expression was regulated by E2, but not P4.

Conclusions

We indicated that cyclic changes in HURP expression in human normal ESC strongly suggested up-regulation by estrogen. Taken together, since estrogen responses are fundamental in endometrial biology, uterine expression of HURP may be involved in female reproductive function during menstrual cycle.

Keywords; hepatoma upregulated protein, endometrium, endometrial stromal cell, human, uterus, menstrual cycle

Introduction

A satisfactory reproductive outcome requires synchronous growth and maturation of the endometrium throughout the menstrual cycle. Dynamic changes occur in the human endometrium during the menstrual cycle under the influence of estrogen and

progesterone. Each phase of the menstrual cycle is precisely tuned by many known and unknown genes that are regulated by endocrine, paracrine, and autocrine factors. Appropriate endometrial changes are essential for successful embryo implantation. An aberrant endometrial status may lead to implantation failure.

Previous reports determined that hepatoma upregulated protein (HURP), a cell cycle-associated gene, affects endometrial stromal proliferation during implantation [1-7]. Loss of HURP expression in the endometrium led to an implantation defect and infertility [7]. The aforementioned indicates that HURP may have structural and functional roles in the female reproductive tract. However, current knowledge regarding HURP's role in the endometrium is mostly derived from research on malignant tissues [1,7–12]. As such, the regulation and possible roles of HURP in human endometrium are uncertain.

This study further defined the role of HURP in human endometrium by characterizing changes in HURP mRNA and protein abundance, as well as the cellular and subcellular localization of HURP protein over the normal human menstrual cycle. In addition, we examined the effect of the steroid hormones E2 and P4 on the expression of HURP in endometrial stromal cells.

Materials and Methods

Patients and samples

Endometrial tissue was obtained from 52 women, either by curettage under sterile conditions or during hysterectomy for benign gynecologic diseases. Samples were collected at the time of hysterectomy, laparoscopic sterilization, or hysteroscopy. Tissue samples were divided into five groups, based on well-established criteria, for the evaluation of mRNA and protein [13]: early-proliferative (n = 12), late-proliferative (n = 14), early-secretory (n = 9), mid-secretory (n = 9), and late-secretory phase (n = 8). Fresh tissue was obtained from biopsy samples and stored at –70°C for Western blotting. It was obtained from biopsies and stored in RNAlater® (Ambion, Austin, TX, USA), according to the manufacturer's instructions for real-time polymerase chain reaction (RT-PCR). The mean (± standard deviation) age of the women was 35.61 ± 7.21 years. All women had regular menstrual cycles, which ranged from 25 to 35 days, and none received hormonal treatment within the 6 months prior to surgery. The experimental procedures were approved by the Institutional Review Board at the Shiga University of Medical Science (approval number 23-72-1). All women provided written informed consent for the use of their endometrial tissues.

Immunohistochemistry

Immunohistochemical staining for was undertaken using the streptavidin-biotin peroxidase (ABC) method using the VECTASTAIN ABC Kit (Vector Laboratories, Burlingame, CA) for peroxidase (rabbit IgG) (Vector laboratories, Burlingame, USA) according to the manufacturer's protocol with rabbit polyclonal anti-human HURP antibody (ab5068, Abcam, Cambridge, MA, UK) at a dilution of 1 out of 200 in TBS/20% swine serum (SS). DAB (Vector Laboratories) were used to visualize the bound antibody. Sections were counterstained with Harris hematoxylin. Negative controls were used to block peptides (ab111970, Abcam).

Isolation and culture of human endometrial stromal cells

Human human endometrial stromal cells (ESCs) were isolated and cultured as previously described [14]. At first passage, the cells were plated in 6-well culture plates (Becton Dickinson) at a density of 1×10^5 cells/mL. Cells were cultured with 4-mL DMEM supplemented with 10% charcoal-stripped fetal bovine serum, 1% antibiotics (Life Technologies), and some other materials, including 17β-estradiol (E2; Sigma) and 4-pregnene-3,20-dione (P4; Sigma) at 48 h.

Western blot analysis

Expression of HURP protein was analyzed by Western blotting as previously described [15]. The primary antibodies used included rabit polyclonal human anti HURP antibody (1:2000) and GAPDH (1:5000). The ECL Western Blotting Detection System was used to detect signals, which were visualized using LAS-4000 mini (GE Healthcare, Little Chalfont, UK). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the loading control.

Total RNA extraction and quantitative real-time PCR

Total RNA was extracted from the samples and purified using TRIzol Reagent with the PureLink™ Mini Total RNA Purification System (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan) was used to synthesize complementary DNA. PCR samples were prepared up to a final volume of 20 μL using 1× SYBR Green Master Mix (Roche, Laval, Canada). Primer sequences used were 5′-GAAAGCAGGAGCAGCATAGAAGA-3′ (sense) and 5′-CACCAGCAAGAAGAGGCAAAC-3′ (antisense) for HURP and 5′-

AAATCCCATCACCATCTTCCA-3′ (sense) and 5′-AATGAGCCCCAGCCTTCTC-3′ The PCR cycling conditions were as follows: 95 °C for 5 min amplification, 40 cycles of 94°C for 15 s, 60°C for 45 s, and 72°C for 10 s on the Roche LC480 system (Roche) Cycle threshold (Ct) values were used to compute levels of mRNA expression from the standard curve. Analytical data were adjusted based on the mRNA expression of GAPDH as an internal control.

Statistical analysis

Since the data from Western blot and mRNA analyses in the endometrium were not normally distributed, they were analyzed via nonparametric analysis of variance on ranks (Kruskal–Wallis test). SPSS ver. 20 (SPSS Inc., Chicago, IL, USA) was used to perform statistical calculations. Statistical significance was defined as $P < 0.05$.

Results

Localization of HURP expression in human endometrium using immunohistochemistry

We analyzed tissue sections at different phases of the menstrual cycle (early-, late-proliferative phase or early-, mid-, late-secretory phase), using immunohistochemistry with an anti-HURP antibody, to assess the subcellular localization of expression of HURP in the endometrial tissue. Immunohistochemistry studies showed diffuse staining in the nucleus of the endometrial epithelial and stromal cells in all stages. Endometrial HURP was weakly expressed in the nucleus of endometrial stromal cells in the early-proliferative phase than in the other phases. In addition, HURP was highly expressed in the mid- and late-secretory phases in the glandular epithelial cells when compared to the other stages (Figure 1). From above, HURP was expressed in both endometrial stromal cells and epithelial cells of human endometrium.

Pattern of endometrial HURP expression

We divided the endometrial tissues into five groups, early-proliferative, late-proliferative, early-secretory, mid-secretory, and late-secretory phase, according to the most recent menstrual period and histology according to the criteria of Noyes et al. [16]. Figure 2 shows that the relative expression of the HURP mRNA level in endometrial tissues peaked in the late-proliferative phase. The expression was also significantly higher in the late-proliferative phase than that in the late-secretory phase. Furthermore,

HURP mRNA was downregulated during the early-, mid-, and late- secretory phases of the menstrual cycle. In accordance with mRNA levels, the protein level of HURP was significantly upregulated during the late-proliferative phase of the menstrual cycle than during the early-proliferative phase. Unlike the expression of HURP mRNA during the secretory phase, the expression of HURP protein levels decreased rapidly in the mid-secretory phase. These results confirm and extend previous murine observations by significantly increasing the sample number and using a histopathological classification instead of a molecular classification of the menstrual cycle.

Steroid hormone regulation of HURP protein expression

We assessed the effects of sex steroid hormones on the regulation of HURP in early-proliferative endometrial stromal cells after 48 h. HURP protein expression was significantly stronger in explants cultured with E2 alone than with the control, P4, and E2 and P4 (Figure 3A, 3B). There were no significant differences in HURP protein expression between the other groups. A panel of chemicals comprising of E2 and P4 was also applied to ESCs at increasing concentrations (E2: 10^{-9} M to 10^{-7} M and P4: 10^{-7} M to 10^{-5} M). A significant increase in the expression of HURP protein was observed in cells treated with E2 at 10^{-8} and 10^{-7}M than with controls (Figure 3C, 3D). However,

we observed no change in the expression of HURP protein cultured with P4 than with controls. Therefore, HURP expression was regulated by E2, but not by P4.

Discussion

The present study showed that the expression of HURP in the endometrium dynamically changes with the menstrual cycle. Also, E2 increased HURP expression in cultured endometrial stromal cells, whereas P4 suppressed its expression. Previous reports revealed that estrogen upregulated HURP expression in murine endometrial stromal cells and that HURP deficiency in murine endometrium was involved in implantation defects [7]. Therefore, this finding supports the possibility that HURP expression may also be associated with impaired implantation in humans.

The present study provides comprehensive information about the dynamic endometrial expression of HURP in the subsequent phases of the same menstrual cycle. A previous study determined that HURP expression was regulated by sex steroid hormones in the murine uterus [13]. The cyclic changes in the endometrium are important for implantation. It is well known that the maturation of the endometrium is important for successful embryo implantation [17,18]. Our results showed the presence of HURP in human endometrium, with an increase in HURP expression in the late-

proliferative phase compared with the early-proliferative phase. The mRNA and protein levels of HURP expression have been maintained in the secretory phase. On the other hand, it has been reported that HURP protein is expressed only in murine endometrial stromal cells [13]. However, we interestingly confirmed that HURP protein was expressed in both endometrial gland epithelial and stromal cells in humans (Figure 1). Therefore, we suggest that HURP might have a role in maintaining the endometrium, although the expression pattern of HURP is different between murine and human endometrium.

We subsequently indicated that the level of HURP protein was increased by 17β-estradiol (E2) in human endometrial stromal cells, and this suppressed the effect of promoting E2 expression by 4-pregnene-3,20-dione (P4). Sex steroid hormones can induce changes in the structure and function of the uterus and regulate menstrual cycle progression. Progesterone can antagonize the actions of estrogen in many ways [19–21]. In the previous study, HURP was shown to be induced by E2 in the mouse uterus via an estrogen-dependent mechanism [13]. In this study, endometrial stromal cells were examined for the effect of E2 and P4 on HURP expression. This study revealed that E2 treatment continually elevated the expression of HURP protein in endometrial stromal cells, while P4 did not change this expression. Simultaneous administration of E2 and

P4 offsets the effect of E2, which increased HURP (Figures 3 and 4). Therefore, this fact might explain why high levels of HURP expression are maintained from the late-proliferative to the late-secretory phase of human endometrial tissue and that HURP is involved in embryo implantation.

A limitation of the present study is that it has a small sample size in each of the subgroups. However, previously published studies using an endometrial biopsy had approximately 60 samples. As such, this study has a relatively large sample size. This study also used samples from groups that did not match the patient characteristics, such as age, BMI, pregnancy history, and history of infertility. However, it is very difficult to recruit women in all subgroups in other studies; therefore, patient characteristics have not been matched. In addition, to the best of our knowledge, this is the first report on HURP expression levels in human endometrium during various phases of the menstrual cycle. Inclusion of an even larger number of patients and evaluation of HURP will hopefully lead to safer conclusions regarding the potential etiopathogenetic role of embryo implantation.

In conclusion, we showed the temporal and spatial expression of HURP in human endometrium. We indicated that cyclic changes in HURP expression in normal human ESC strongly suggest upregulation by estrogen. Given that estrogen responses

are fundamental in both endometrial biology and female reproduction, it is possible that HURP is involved in these processes. The relationship between HURP expression and implantation failure should be explored.

Acknowledgments

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Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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Figure legends

Figure 1. Immunolocalization of HURP protein in the human endometrium

HURP protein was localized in the nucleus of the endometrial epithelial and stromal cells in all stages. Original magnification ×100. (A, C, E, G, I) Immunostaining with the primary antibody for HURP. (B, D, F, H, J) Addition of blocking peptides for HURP to the primary antibody for HURP. (EP, early-proliferative phase; LP, late-proliferative phase; ES, early-secretory phase; MS, mid-secretory phase; LS, late-secretory phase)

Figure 2. Expression of HURP mRNA and protein levels in human endometrium in various stages of the menstrual cycle

Endometrial tissues were divided into five groups, early-proliferative (EP), late-proliferative (LP), early-secretory (ES), mid-secretory(MS), late-secretory (LS) phase, based on the endometrial tissues classified according to the last menstrual period and histology according to the criteria of Noyes et al. [16]. (A) Uterine HURP mRNA expression during the menstrual cycle was examined by quantitative RT-PCR. A significant increase in HURP expression level was observed during the menstrual cycle ($^{*}P < 0.05$, $^{**}P < 0.05$ vs. EP). (B) Twenty micrograms of proteins were immunoblotted with a HURP polyclonal antibody (upper panel). This membrane was

also reprobed with an anti-actin antibody, which was used as an internal control to indicate relative loading of the samples (lower panel). (C) Western blot was used to examine uterine HURP protein expression during the menstrual cycle. A significant increase in HURP expression level was observed during the menstrual cycle ($^{*}P < 0.05$ vs. EP, LP, ES, and MS.).

Figure 3. Effect of sex steroid hormones E2 and P4 on regulation of HURP protein expression

A), B) The cells were treated with DMSO (C, negative control), E2 (10^{-8}M), P4 (10^{-6}M) and combination of E2 (10^{-8}M) and P4 (10^{-6}M) for 2 days and harvested 24 h following the final injection. C), D) The cells were treated with DMSO (C, negative control), E2 ($10^{-9 \text{ to } -7}$M), P4 ($10^{-7 \text{ to } -5}$M) for 2 days and harvested 24 h following the final injection. Graphs represent the analysis of immunoblotting data. (*P < 0.05 vs. control)

Figure 1　Immunolocalization of HURP protein in the human endometrium

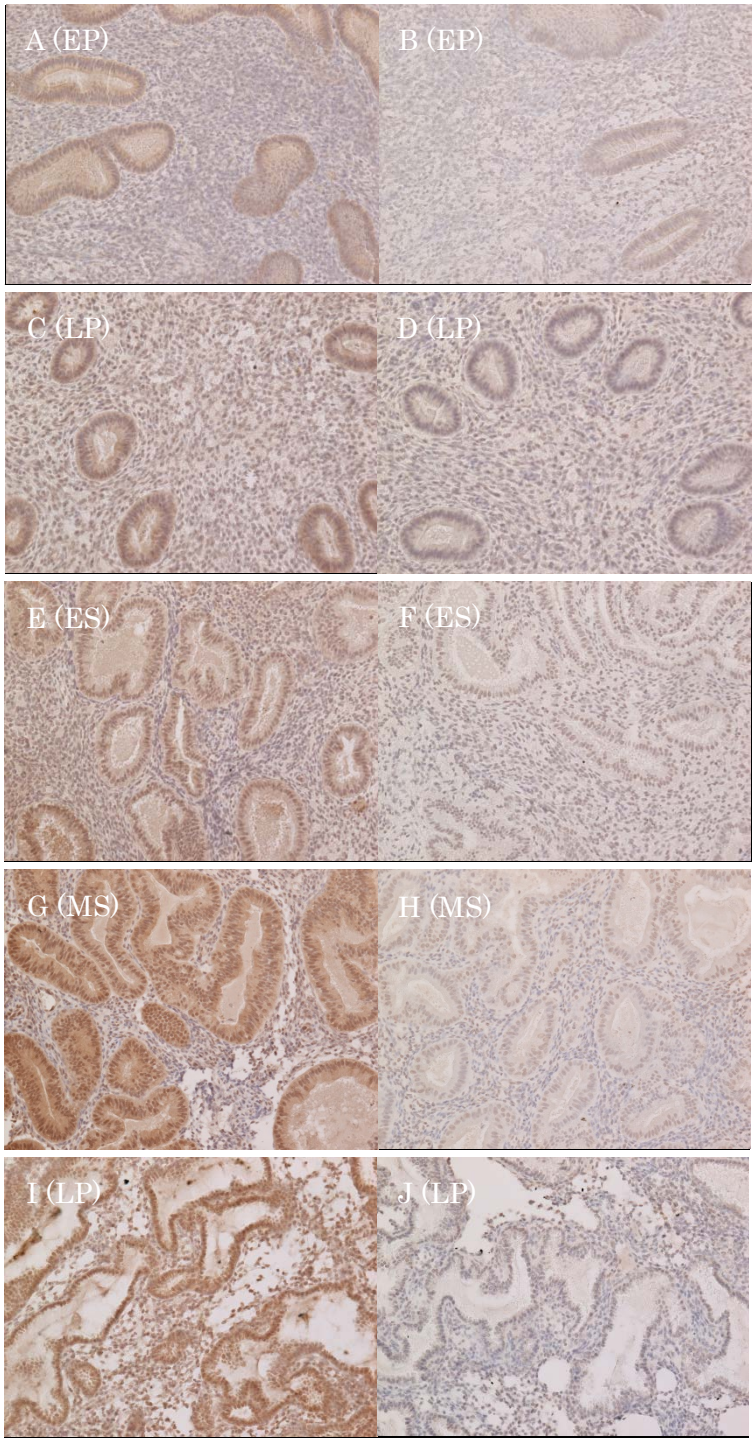
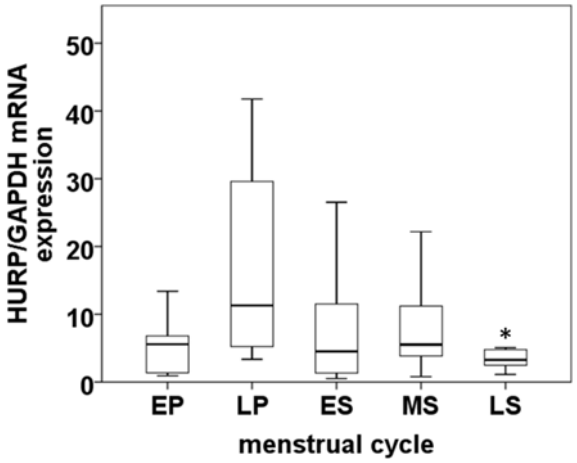


Figure 2 Expression of HURP mRNA and protein levels in various stages of human endometrium.

A



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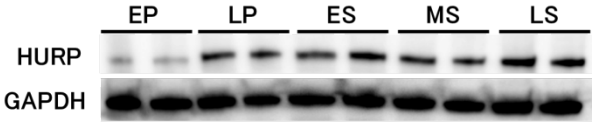


C

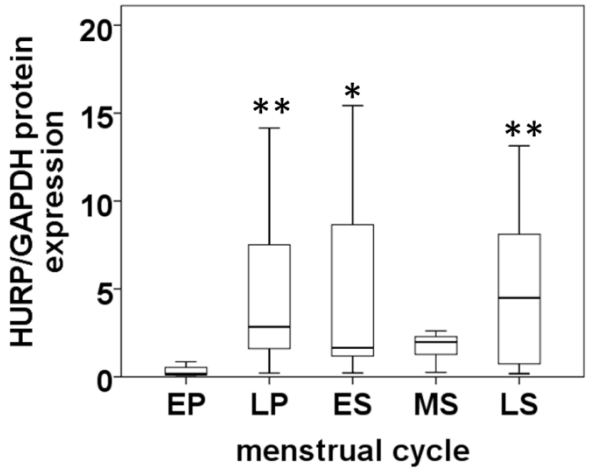
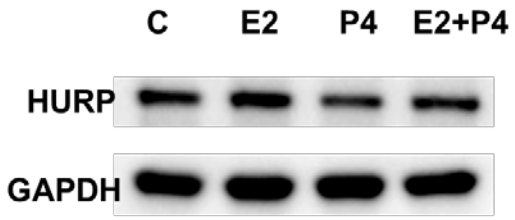


Figure 3 Effect of sex-steroid hormones E2 and P4 on regulation of HURP protein expression

A



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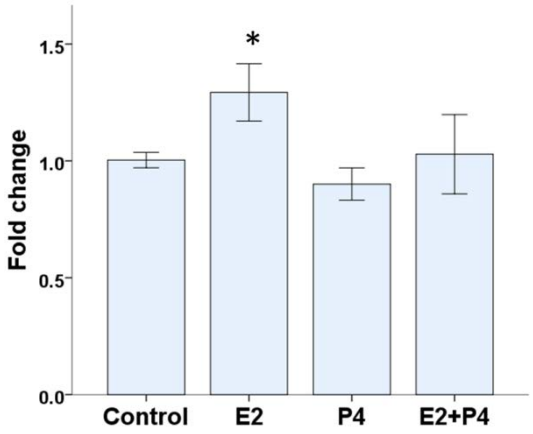

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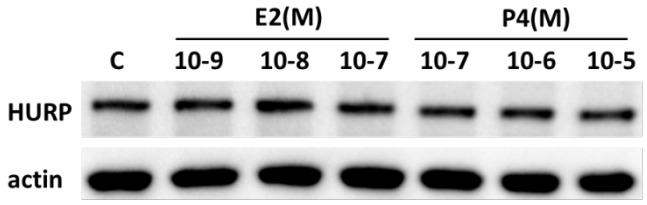


D

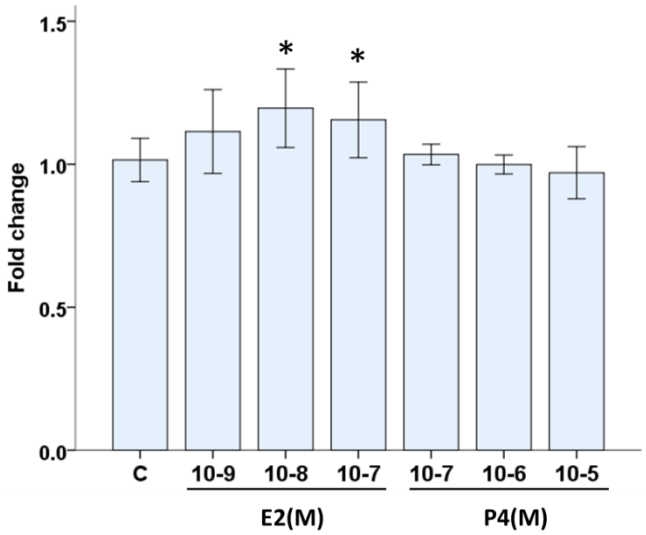
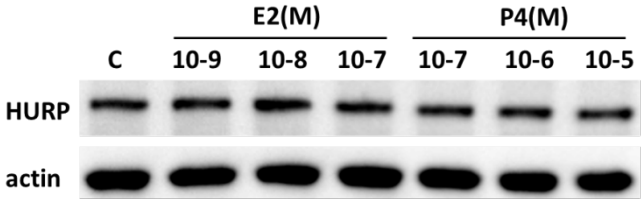

1.5
1.0
0.5
0.0
Fold change
*
*
C
10-9
10-8
10-7
10-7
10-6
10-5
E2(M)
P4(M)

Figure 4 Effect of sex-steroid hormones E2 and P4 on regulation of HURP protein expression

A



B