The mRNA-Binding Protein HuR is Regulated in the Menstrual Cycle and Repressed in Ectopic Endometrium

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Abstract

Cytokines modulate turnover of the endometrium during the menstrual cycle and contribute to the pathogenesis of endometriosis. Gene expression for cytokines is often regulated by proteins that bind to adenosine- and uridine-rich elements (AREs) in their transcripts to stabilize or destabilize bound messenger RNAs (mRNAs). HuR/ELAVL1 is an RNA-binding protein that stabilizes ARE-containing mRNAs. We hypothesized that HuR might play a role in regulating cytokine expression during the menstrual cycle and in endometriosis and characterized the expression and regulation of HuR in eutopic and ectopic human endometrium. Tissue sections obtained from normal ($n = 23$) and ectopic ($n = 16$) endometrium were immunostained for HuR, and staining intensity was evaluated by HSCORE. Cultured stromal cells isolated from normal endometrium were treated with vehicle, estradiol (E2), progesterone (P), E2 + P, tumor necrosis factor- α (TNF- α), and interleukin 1 β (IL-1 β) for 24 hours, and HuR expression was determined by Western blot. HuR immunoreactivity was significantly lower in the early proliferative and late secretory phases (157.5 ± 11.08 and 190.0 ± 15.2 , respectively), compared to the mid-late proliferative (270.0 ± 8.0) and early-mid secretory phases (256.6 ± 20.2 ; $P < .01$, analysis of variance [ANOVA]). Furthermore, HuR expression was significantly lower in ectopic endometrial cells compared to normal endometrium in mid-late proliferative and early-mid-secretory phases ($P < .01$). Estrogen, P, or cytokines did not alter HuR expression in cultured endometrial stromal cells. Increased HuR levels in the mid-menstrual phases are likely to contribute to reduced mid-cycle cytokine expression and enhanced cellular survival in eutopic endometrium. In ectopic endometrium, elevated cytokine levels associated with endometriosis likely reduce HuR expression.

Keywords

endometrium, endometriosis, HuR, cytokine

Introduction

The endometrium, the innermost layer of uterus, comprises hormonally responsive glandular epithelia and stroma as well as large numbers of bone marrow-derived leukocytes that collectively permit embryo implantation and development. Endometrial cells respond to sex steroids, principally estradiol (E2) and progesterone (P), to undergo extraordinary growth and regression in a cyclic manner. This tissue will be shed and regenerated ~ 450 times during the reproductive lifetime of a woman. The functional layer of the endometrium is involved in unique functions including blastocyst implantation, immunological tolerance of the partial allograft (ie, the embryo), regulation of placental trophoblast invasion, protection against ascending infection, and efficient disposal of blood and cellular debris during menstruation when embryo implantation does not occur.

Immune cells and mediators play critical roles in endometrial function. Macrophages serve as scavengers, helping to clear apoptotic cells and cellular debris. T-lymphocytes promote

B cell production of antibodies and also regulate macrophage functions. Along with resident leukocytes, endometrial stromal and glandular cells produce chemokines (chemotactic cytokines) including, tumor necrosis factor- α (TNF- α), interleukin 1 β (IL-1 β), interleukin 8 (IL-8), monocyte chemotactic protein 1 (MCP-1), and Regulated upon activation, Normal

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T cell Expressed and Secreted (RANTES).¹⁻⁴ In addition to regulating leukocyte migration and function in preparation for embryo implantation, these small peptides regulate the process of apoptosis of endometrial stromal and glandular cells during menstruation.²

The presence of endometrial stromal and glandular cells outside of the uterus (ie, ectopic endometrium) represents a pathologic condition termed endometriosis. Endometriosis most commonly presents with pelvic pain and/or infertility and affects 7% to 10% of women in the general population with a prevalence of 0.5% to 5% in fertile and 25% to 40% in infertile women.⁵ Chronic pelvic pain and infertility associated with endometriosis lead to significant financial burden for the individual and society.⁶ Multiple theories have been proposed to explain the pathogenesis of endometriosis. Among these, the most commonly accepted remains Sampson's theory of retrograde menstruation from the uterus, through the fallopian tubes, and into the pelvic cavity.⁷⁻⁹ Because retrograde menstruation occurs in 76% to 90% of women with patent fallopian tubes,^{10,11} it is recognized that additional factors must play a role in determining susceptibility to endometriosis.

A multitude of immunologic abnormalities have been observed in women with endometriosis and have indicated that an altered immune response may lead to inadequate removal of refluxed menstrual debris and/or promote the growth of ectopic endometrium in susceptible women.⁹ Endometriosis is associated with changes in both cell-mediated and humoral components of innate and acquired immunity. In addition to changes in the quantity and activity of immune cells, endometriosis has been associated with altered expression of cytokines and growth factors, which may promote implantation and growth of ectopic endometrium by inducing cellular proliferation and angiogenesis.² Several cytokines and growth factors are elevated in the peritoneal fluid of women with endometriosis, including IL-1,^{12,13} IL-8,^{14,15} MCP-1,¹⁶ RANTES,¹⁷ TNF- α ,¹⁸ and vascular endothelial growth factor (VEGF).¹⁹

Gene expression for many cytokines is regulated posttranscriptionally, at the level of messenger RNA (mRNA) stability.²⁰ Much of this regulation occurs by the binding and stabilizing, or destabilizing of these mRNAs by proteins that recognize Adenosine- and Uridine-rich elements (ie, AU-rich elements or AREs) in 3' untranslated regions of the transcripts.^{21,22} ARE-binding proteins alter the stability of their target mRNAs and/or regulate target mRNA translation by binding to core and flanking sequences of these AU-rich elements and then interacting with protein complexes essential for mRNA degradation or translation.²³

HuR/ELAVL1 is an ARE-binding protein that regulates proliferation and stress responsiveness by regulating transcripts such as p21, cyclin A, cyclin B1,^{24,25} as well as regulating mRNAs encoding transcription factors such as FOS.²⁶ In addition, HuR promotes the mRNA stability of a large number of ARE-bearing transcripts encoding inflammatory and immune factors, including TNF- α ,²⁷ IL-1 β ,²⁸ IL-3,²⁹ IL-8,³⁰ granulocyte monocyte colony stimulating factor (GM-CSF),²⁹

cyclooxygenase 2 (COX-2),³¹ VEGF,²⁹ matrix metalloprotein 9 (MMP-9),³² CD154 (the CD40 ligand),³³ and β -adrenergic receptors.³⁴ In summary, HuR is a stabilizing factor that selectively binds and enhances the expression of target mRNAs including many that encode for cytokines.³⁵

We have established that HuR is expressed in human endometrium, and we hypothesized that HuR might play a role in regulating the changes in cytokine expression required for normal menstrual cyclicity. Given the cytokine-rich environment of endometriosis, we reasoned that altered HuR expression might play a role in the pathogenesis of this condition. To this end, we have characterized the expression of HuR in eutopic and ectopic human endometrium in vivo and have studied the regulation of HuR using primary endometrial stromal cell cultures.

Materials and Methods

Tissue Collection

All endometrial tissue samples were collected at Yale University School of Medicine. The study was approved by the human investigation committee. Eutopic endometrial tissues samples were obtained from human uteri after hysterectomy, or from endometrial biopsies, in all cases conducted for reasons that exclude suspected endometrial disease. Menstrual cycle dating was established from the participant's menstrual history and was confirmed by endometrial histology using the criteria of Noyes et al.³⁶ Eutopic endometrial tissue samples from 23 women (mean age 35.7) were used for histochemical analysis and were grouped according to menstrual cycle phase: early proliferative ([EP] days 1-5, $n = 5$), mid-proliferative ([MP] days 6-10, $n = 2$), late proliferative ([LP] days 11-14, $n = 2$), early secretory ([ES] days 15-18, $n = 5$), mid-secretory ([MS] days 19-23, $n = 6$), and late secretory ([LS] days 24-28, $n = 3$).

To isolate primary endometrial stromal cells for culture, endometrial samples ($n = 5$, mean age, 38 years; range, 30-43 years) were obtained from fertile women who did not receive hormonal treatment in the preceding 3 months. The endometrial samples were placed in Hank balanced salt solution (HBSS; Sigma-Aldrich, St Louis, Missouri) and transported to the laboratory for isolation of human endometrial stromal cells (HESCs) and long-term culture as described below and as previously described.³⁷

Ectopic endometrial tissues were obtained from 16 women with endometriosis (mean age 42.4 years, range 25-53 years) undergoing laparoscopy. All women in the study group had advanced stages of endometriosis (stage III-IV) according to the revised American Fertility Society Classification system.³⁸ None of the patients had undergone hormonal treatment during the 3 months prior to laparoscopy. The presence of endometriosis was confirmed by histopathologic examination of the implants. Although no strict criteria were applied to classify the ectopic lesions as active or inactive, we considered endometriosis samples to be inactive when we observed abundance

of pigmented histiocytes and little endometrial stroma and/or glands, and we excluded these samples from further study as has previously been described.³⁹

Ectopic endometrial samples were grouped based on menstrual history and endometrial histology³⁶ of corresponding eutopic endometrial samples belonging to the same patient obtained by endometrial biopsy at the time of laparoscopy (EP [days 1-5, n = 1], MP [days 6-10, n = 2], LP [days 11-14, n = 4], ES [days 15-18, n = 4], MS [days 19-23, n = 4], LS [days 24-28, n = 1]). For immunohistochemical analysis, samples were fixed in 10% buffered formalin and processed routinely for paraffin embedding (below).

Immunohistochemistry

Paraffin-embedded tissue samples were cut into 5- μ m sections and mounted on SuperFrost Plus Slides (Erie Scientific Company, Portsmouth, New Hampshire). Tissue sections were deparaffinized in xylene, rehydrated in graded series of alcohol, and immersed in distilled water. Antigen retrieval was performed by heating the samples to a boil in citrate buffer solution (10 mmol/L, pH 6.0) in a microwave. Endogenous peroxidase activity was blocked by 3% H₂O₂ in distilled water with 50% methanol at room temperature for 20 minutes, followed by 3 rinses in phosphate buffered saline (PBS) for 5 minutes each. Slides were then incubated with 5% blocking horse serum (LabVision, Fremont, California) in PBS in a humidified chamber for 30 minutes at room temperature. Excess serum was then drained, and sections were incubated overnight at 4°C in a humidified chamber with the monoclonal mouse anti-human HuR antibody (affinity-purified mouse immunoglobulin G [IgG], dilution 1:250; Santa Cruz Biotechnology Inc, Santa Cruz, California). Negative control sections were incubated with the equivalent protein concentration of mouse IgG (Vector Laboratories, Burlingame, California). Sections were then rinsed in tris-buffered saline (TBS), and biotinylated horse anti-mouse antibody (1.5 mg/mL; Vector Laboratories) was added at 1:400 dilution for 45 minutes at room temperature. The antigen-antibody complex was detected using an avidin-biotin-peroxidase kit (LabVision). Diaminobenzidine (3,3-diaminobenzidine tetrahydrochloride dihydrate; LabVision) was used as the chromogen and the sections were counterstained with hematoxylin and eosin mounted with Permount (Fisher Chemicals, Springfield, New Jersey) on glass slides.

Immunohistochemical staining for HuR was evaluated in a semiquantitative fashion (ie, 0 [absent staining] to 3 [most intense staining]). Epithelial and stromal cells were separately evaluated and scored. For each slide, an HSCORE value was derived by summing the percentages of stained cells at each intensity, multiplied by the score of intensity [$HSCORE = \sum Pi (i + 1)$] where *i* is the intensity score and *Pi* is the corresponding percentage of the cells. In each slide, 10 different areas were evaluated under the microscope, and the percentage of the cells for each intensity within each of these areas was determined at different times by 2 investigators blinded to the type

and source of the tissues. The average of the 2 independent scores was used.

Isolation and Culture of HESCs

Human Endometrial Stromal Cells were separated and maintained in monolayer culture as previously described.³⁷ Briefly, endometrial tissue was minced with a sterile surgical blade and digested in HBSS (Sigma-Aldrich) containing collagenase B (1 mg/mL, 15 U/mg; Roche, Indianapolis, Indiana), deoxyribonuclease I (0.1 mg/mL, 1500 U/mg; Roche), penicillin (200 U/mL), and streptomycin (200 mg/mL) for 45 to 60 minutes at 37°C with agitation. The dispersed endometrial stromal cells were separated by filtration through a wire sieve (73- μ m diameter pore; Sigma-Aldrich) and cultured in Dulbecco modified Eagle's medium (DMEM) Ham F-12 (1:1 v/v; Sigma-Aldrich) containing fetal bovine serum (FBS, 10% v/v; Gibco/BRL, Rockville, Maryland). Cells were plated in plastic flasks (75 cm²; Falcon, BD Biosciences, Franklin Lakes, New Jersey). The cultures were maintained in a standard 95% air: 5% CO₂ incubator at 37°C and allowed to replicate to confluence. Cells were passed by standard methods of trypsinization, plated in DMEM onto 60-mm culture dishes, and grown to pre-confluence. Prior to treatment with steroids or cytokines, HESCs were incubated in serum-free, phenol red-free media (Sigma-Aldrich) for 24 hours.

Cell Culture

Cultured HESCs were treated with vehicle, E2 (10⁻⁸ mol/L), progesterone ([P4], 10⁻⁸ mol/L), E2 (10⁻⁸ mol/L) + P4 (10⁻⁸ mol/L), or TNF- α (10 ng/mL) + IL-1 β (10 ng/mL) for 24 hours. Following treatments, media were removed, plates were rinsed in PBS, and stored at -80°C until analyzed.

Western Blot Analysis

Total protein from treated HESC was extracted with tissue protein extraction reagent (T-PER) (Pierce, Rockford, Illinois) supplemented with a protease inhibitor cocktail (1 mmol/L Na₃VO₄, 10 μ g/mL leupeptin, 10 μ g/mL aprotinin, and 1 mmol/L phenylmethylsulfonylfluoride [PMSF]; Calbiochem, San Diego, California). Protein concentrations were determined by detergent-compatible Bradford protein assay (Bio-Rad laboratories, Hercules, California). Western blot analysis was performed as described previously.⁴⁰ Briefly, 10 μ g of protein was loaded into each lane, separated electrophoretically by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% Tris-HCl Ready Gels (Bio-Rad Laboratories), and electroblotted onto a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was blocked with 5% nonfat dry milk in TBS-T buffer (0.1% Tween-20 in Tris-buffered saline) for 1 hour at room temperature to reduce nonspecific binding. The membrane was then incubated with primary antibody (monoclonal mouse antihuman HuR antibody [dilution 1:2500, Santa Cruz Biotechnology Inc], or

Figure 1. HuR protein expression in representative examples of eutopic endometrial tissue from early proliferative (A), mid-proliferative (B), early secretory (C), and late secretory (D) phases of the menstrual cycle. HuR immunostaining is predominantly nuclear. HuR protein expression is significantly lower in the late secretory and early proliferative phases compared to the mid-proliferative and early secretory phases.

monoclonal mouse antihuman IL-8 antibody [dilution 1:300, R&D Systems, Minneapolis, Minnesota], or polyclonal rabbit anti-human ER α antibody [dilution 1/500, Santa Cruz Biotechnology]) overnight at 4°C, and washed 3 times with TBS-T for 20 minutes. Then, the membrane was incubated for 1 hour at room temperature with secondary antibody (peroxidase-labeled anti-mouse IgG for HuR and IL-8, or peroxidase-labeled anti-rabbit IgG for ER α [both at 1:5000 dilution, Vector Laboratories]) and subsequently washed with TBS-T 3 times for 20 min/wash. HuR levels were detected using chemiluminescent detecting reagents (Perkin Elmer Life Sciences, Boston, Massachusetts) using BioMax film (Kodak, Rochester, New York).

Membranes were then stripped with stripping solution (Pierce) prior to re-probing with anti-human β -actin antibody. Immunoblot bands were quantified using a laser densitometer. Each HuR band was normalized to the value obtained from the corresponding β -actin band.

Statistical Analyses

Because the data from Western blot analyses were normally distributed (as determined by the Kolmogorov-Smirnov test), comparisons of samples were analyzed with Student *t* test or one-way analysis of variance (ANOVA) followed by a post hoc Holm-Sidak test where appropriate. Because

Figure 2. A, HuR protein expression in eutopic endometrial glands according to the menstrual cycle phase. HSCORE analysis was performed to quantitate endometrial glandular expression of HuR from biopsy samples. B, HuR protein expression in eutopic endometrial stromal cells according to the menstrual cycle phase. HSCORE analysis was performed to quantitate endometrial stromal cell expression of HuR from biopsy samples. EP indicates early proliferative (n = 5); MP-LP, mid-proliferative + late proliferative (n = 4); ES-MS, early secretory + mid-secretory (n = 11); LS, late secretory (n = 3).

immunohistochemistry scores in the endometrium were not normally distributed, these data were analyzed with nonparametric ANOVA on ranks (Kruskal-Wallis test). Statistical calculations were performed using SigmaStat for Windows, version 3.0 (Jandel Scientific Corp, San Rafael, California). Statistical significance was defined as $P < .05$.

Results

HuR Protein Expression in Human Endometrium is Temporally Regulated During the Menstrual Cycle

A total of 23 endometrial samples obtained from women without known endometrial disease or endometriosis were evaluated by immunohistochemistry. The distribution according to menstrual cycle phase was EP (days 1-5, n = 5), mid- and late-proliferative (MP-LP, days 6-14, n = 4), ES-MS

(days 15-23, n = 11), and LS(days 24-28, n = 3). Although ES-MS phases of the endometrium are structurally and functionally distinct, samples from these phases showed similar HuR expression and these data were pooled. Combining these samples allowed us to compare fewer groups each of greater sample size. Mid-proliferative and LP endometrial samples were grouped together for the same reasons.

Immunohistochemical analysis of the endometrial sections revealed predominantly nuclear staining of the glandular and stromal cells throughout the menstrual cycle (Figure 1). HuR immunoreactivity in both glandular and stromal cells was significantly lower in the LS and EP phases compared to MP-LP and ES-MS phases ($P < .05$, Figures 1, and 2A, B). There was negligible staining in negative control samples using nonspecific mouse IgG (not shown).

HuR Protein Expression is Decreased in Ectopic Compared With Eutopic Endometrium

Endometriotic tissue was morphologically heterogeneous and, compared with the normal endometrium, the glands appeared larger and architecturally disorganized. HuR immunostaining was principally nuclear in all ectopic endometrial tissues. Mean staining intensity in stromal and glandular cells was compared between normal endometrium and endometriosis samples from each participant (n = 16). Mean HSCORE values for stromal and glandular HuR expression were lower in the endometriosis samples from MP-LP and ES-MS phases compared with normal endometrium from the same phases ($P < .05$, Figures 3 and 4). Number of samples was not adequate for comparison of EP and LS cycle phases.

HuR Protein Expression in HESCs is Not Directly Regulated by Estrogen or Progesterone

Given our observation that there is menstrual-cycle-dependent expression of HuR in vivo, we tested whether the critical sex steroids that dictate endometrial (and endometriotic) functions (estrogen and progesterone) also regulated the expression of HuR in isolated HESCs. Human endometrial stromal cell preparations were treated with vehicle (n = 4), E2 (10^{-8} mol/L; n = 4), P4 (10^{-8} mol/L; n = 4), or E2 + P4 (10^{-8} mol/L each; n = 4) for 24 hours. Western blot analyses were performed to evaluate HuR protein levels. Endometrial stromal cells treated with E2, P4, or E2 + P4 did not show a significant change in HuR expression when compared to those treated with vehicle alone (Figure 5A). Expression of ER α in cultured endometrial stromal cells was confirmed by Western blot analysis using tissue cultured from 2 different patients (Figure 5B).

HuR Protein Expression in HESCs is Not Directly Regulated by the Cytokines TNF- α and IL-1 β

We then investigated whether inflammatory cytokines might be responsible for the regulated expression of HuR throughout the menstrual cycle. Primary HESCs were treated with either

Figure 3. Representative micrographs of HuR protein immunostaining in eutopic endometrium in the late proliferative phase (A) and in matched ectopic endometrium from the late proliferative phase (B). HuR expression is nuclear in both glandular and stromal components of normal endometrium and ectopic endometrium in all menstrual phases.

Figure 4. HuR protein expression in matched eutopic and ectopic endometrial tissues according to the menstrual cycle phase. HSCORE analysis of HuR expression in HESCs and HEGCs is shown. Significantly lower levels of HuR immunoreactivity were detected in HEGCs (left panel) and HESCs (right panel) from mid-late proliferative and early mid-secretory phases of ectopic endometrium compared to levels detected from normal endometrium in the same menstrual cycle phases. MP-LP indicates mid-proliferative + late proliferative (n = 6); ES-MS, early secretory + mid-secretory (n = 8); (HESCs), human endometrial stromal cells. HEGCs, human endometrial glandular cells.

vehicle (n = 3) or TNF- α (10 ng/mL) plus IL-1 β (10 ng/mL; n = 3) for 24 hours. HuR levels were analyzed by Western blot, which revealed that HuR expression was not altered by cytokine exposure in isolated endometrial cell cultures (Figure 5C). To confirm that our in vitro endometrial stromal cell culture system is responsive to cytokine treatments, we tested whether treatment with TNF- α and IL-1 β increased

IL-8 expression as previously reported.¹⁵ Indeed, treatment with TNF- α and IL-1 β resulted in more than 5-fold increase in IL-8 expression (Figure 5D).

Discussion

In this study, we have characterized the expression of the ARE-binding protein HuR in human eutopic and ectopic endometrial tissues. We have also tested the ability of sex steroids and cytokines to regulate HuR expression in isolated HESCs. Previous work has suggested a role of immune factors in both the cyclic regulation of endometrial functions and in the pathogenesis of endometriosis. Our findings suggest that HuR may play a role in normal endometrial physiology and in the development or maintenance of ectopic endometrial implants in endometriosis.

Cytokines are critical regulators of leukocyte migration and function during the endometrial cycle. In addition, they mediate apoptosis of endometrial glandular and stromal cells during menses. Endometrial cells express a large number of cytokines including IL-8, MCP-1, and RANTES, which recruit immune cells such as T lymphocytes, monocytes, basophils, and eosinophils.⁴¹ Many of these cytokines are most highly expressed in the endometrium during the perimenstrual period of the menstrual cycle coincident with the need for leukocyte infiltration.⁴²

Because several of the cytokines/chemokines that are expressed in the endometrium are reported targets of HuR in alternative cell types,^{27,28,30} and because cytokine expression is temporally regulated during the menstrual cycle, we first examined whether HuR is expressed in human endometrium

Figure 5. A, HuR protein expression in normal primary human endometrial stromal cells treated with estradiol (E2) and progesterone (P). Cultured endometrial stromal cells were treated with vehicle ($n = 4$), E2 (10^{-8} mol/L; $n = 4$), P4 (10^{-8} mol/L; $n = 4$), or E2 + P4 (10^{-8} mol/L each; $n = 4$) for 24 hours. Total protein was extracted and HuR protein was analyzed by Western blot. A band corresponding to HuR was observed at ~ 36 kDa. Re-probing the blot with an antibody against β -actin provided internal loading control. Bars represent means plus SEM. P = nonsignificant for control vs E2, P4, E2 + P4. B, ER α protein expression in normal untreated primary human endometrial stromal cells. Total protein was extracted from cultured endometrial stromal cells ($n = 2$) and ER α protein was analyzed by Western blot. A band corresponding to ER α was observed at ~ 65 kDa. Re-probing the blot with an antibody against β -actin provided internal loading control. C, HuR protein expression in normal primary human endometrial stromal cells treated with TNF- α plus IL-1 β . Cultured endometrial stromal cells from both phases were treated with either vehicle ($n = 3$), or 10 ng/mL TNF- α plus 10 ng/mL IL-1 β ($n = 3$) for 24 hours each. Total protein was extracted and HuR protein was analyzed by Western blot. A band corresponding to HuR was observed at ~ 36 kDa. Re-probing the blot with an antibody against β -actin provided internal loading control. Bars represent means plus SEM. P = nonsignificant comparing control vs cytokine treated cells. D, IL-8 protein expression in normal primary human endometrial stromal cells treated with TNF- α plus IL-1 β . Cultured endometrial stromal cells were treated with either vehicle ($n = 1$) or 10 ng/mL TNF- α plus 10 ng/mL IL-1 β ($n = 1$) for 24 hours. Total protein was extracted and IL-8 protein was analyzed by Western blot. A band corresponding to IL-8 was observed at ~ 8 kDa. Re-probing the blot with an antibody against β -actin provided internal loading control. Treatment with TNF- α plus IL-1 β resulted in an increase in IL-8 expression, demonstrating that our model system is responsive to these cytokines. SEM indicates standard error of mean; IL, interleukin; TNF, tumor necrosis factor.

and whether its expression varies with the phases of the menstrual cycle. HuR was detectable in human endometrial stromal and glandular cells. HuR protein expression was significantly lower in the LS and EP phases compared to MP-LP and ES-MS phases, consistent with a role of HuR in promoting cellular survival and preventing apoptosis.⁴³ Low HuR expression was similarly associated with menstrual phases that demonstrate high inflammatory cytokine expression in the endometrium (prior to and just after the menses). Whether decreased HuR expression during these menstrual phases reflects a compensatory mechanism that buffers the expression of inflammatory cytokines in the endometrium remains to be tested in endometrial cells. Because many cell cycle proteins, cytokines, and growth factors such as leukemia inhibitory factor (LIF) and tumor growth factor- α (TGF- α) produce important changes in the decidual endometrium that are essential to successful embryo implantation, a mid-cycle rise in HuR expression may be an important part of stabilizing these transcripts and enhancing their expression in preparation for the developing embryo.⁴⁴ Although the human LIF transcript has 7 potential AU-rich elements, and TGF- α has 13 AU-rich elements in its 3'-untranslated region, regulation of LIF and/or TGF- α mRNA stability by ARE-binding proteins has not been reported.

Observing that endometrial HuR expression was regulated during the menstrual cycle, we next tested whether steroid hormones play a role in HuR expression in HESCs. Bone morphogenic protein/proteins and nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) regulate HuR expression in renal cells and gastrointestinal tumor cells,^{45,46} respectively, but the regulation of HuR expression in endometrial cells has not yet been investigated. Levels of circulating E2 rise 1 week before ovulation and climb steeply for the last few days of the proliferative phase; a second estrogen peak occurs 5 to 7 days after ovulation. The rise of estrogen in these phases mirrors the observed enhancement of HuR expression that was noted by immunostaining of HuR from primary endometrium. By contrast, progesterone levels rise and remain elevated throughout the luteal phase. The LS and early follicular phases of the menstrual cycle are characterized by low levels of both estrogen and progesterone.⁴⁷

We observed no direct effect of hormone treatments (E2, P, or E2 + P) on HuR expression in isolated HESCs in culture, and we conclude that a direct effect of these steroids on HuR expression is unlikely. In support of this observation, in a computational analysis of the human HuR promoter (interrogating the 2Kb sequence 5' of the transcription start site) for potential transcription factor binding sites, we found no evidence for consensus or degenerate estrogen receptor response elements (EREs) or progesterone receptor response elements (PREs; data not shown). High scoring response elements for NF κ B and Sp1 were noted although the ability of these transcription factors to regulate HuR expression in the endometrium remains to be demonstrated. Furthermore, comparing the current data with gene expression profiling in an alternative but estrogen-responsive cell type, MCF-7 breast cancer cells, we and others

found no evidence for estrogen-dependent expression of HuR.⁴⁸⁻⁵¹ Finally, although we recognize that estrogen receptors perform long-range enhancer functions over hundreds of kilobase (kb) in genomic distance, our genome-wide location analysis for ER α revealed no receptor-bound sites within 200 kb of the *HuR* gene in MCF-7 cells.⁵¹

Therefore, although our study demonstrated that HuR is expressed in human endometrium and that endometrial HuR expression is regulated throughout the menstrual cycle, we were unable to identify the mechanism by which HuR expression is regulated in the endometrium. Our in vitro findings strongly suggest that steroid hormones or cytokines do not play a direct role in this regulation (Figure 5A, C). However, other cytokines/chemokines could potentially be involved. Alternatively, steroid hormones or cytokines could act through intermediary or paracrine factors to secondarily regulate endometrial HuR expression.

In addition to their role in normal endometrial physiology, several chemokines are associated with the development and maintenance of endometriotic implants (ie, endometriosis). Among these, IL-8 levels are elevated in the peritoneal fluid of women with endometriosis and correlate with severity of disease.¹⁵ Interleukin 8 acts as an autocrine growth factor, increases the proliferation of endometrial stromal cells,⁵² and its expression is upregulated by chemokines such as IL-1 β and TNF- α .¹⁵ Similarly, MCP-1 and RANTES levels are elevated in the peritoneal fluid of women with endometriosis^{53,54} and their expression is induced by regulatory chemokines such as IL-1 β .^{55,56}

We observed that both stromal and glandular HuR expression was lower in the endometriosis samples from MP-LP and ES-MS phases compared with matched samples of eutopic endometrium from the same patients. We proposed that HuR expression in ectopic endometrial tissue is decreased in a compensatory response to elevated local cytokine levels such as IL-1 β , IL-8, TNF- α , growth-regulated alpha (GRO- α), RANTES, complement components C3 and C4 each of which is significantly increased in the peritoneal fluid of women with endometriosis.^{8,9} We, therefore, tested whether cytokine levels mediate endometrial HuR expression using an in vitro system of primary HESCs. However, we found that HuR expression in cultured endometrial stromal cells was not directly regulated by IL-1 β plus TNF- α treatment (Figure 5C). We conclude that lower expression of HuR in ectopic endometrium compared to normal eutopic endometrium may not be directly consequent to the effects of elevated cytokine levels associated with endometriosis. The effects of lower HuR expression on gene expression in ectopic endometrium and the contribution of altered HuR expression to the pathogenesis of endometriosis remain to be elucidated.

In the past several decades several pathways of mRNA decay have been described which can converge to influence the stability of particular mRNAs in eukaryotic cells. Many signaling pathways and proteins participate in these processes, some which promote and others which impair mRNA expression. Adenosine- and uridine-rich element (ARE)-mediated mRNA

degradation is one pathway of mRNA decay. HuR antagonizes ARE-mediated transcript degradation²⁹ for a multitude of target mRNAs including those encoding for cytokines/chemokines.²³ Importantly, the mRNA targets of HuR depend on the cell type and cell milieu⁵⁷ and identifying the mRNA targets of HuR in endometrial cells will be important in elucidating a role for HuR in endometrial function and in the pathogenesis of endometriosis.

HuR is also implicated in the regulation of micro-RNA (miRNA)-mediated control of mRNA translation.⁵⁸ Micro-RNAs are a large family of short noncoding mRNAs that repress expression of target genes through sequence complementation, which leads to the degradation of target mRNAs and/or inhibition of their translation.⁵⁹ Micro-RNAs are expressed in human endometrium where their expression is regulated through the menstrual cycle.⁶⁰ HuR relieves the miR-122-induced inhibition of cationic acid transporter 1 (CAT-1) expression in human hepatocarcinoma cells by binding to the 3' untranslated region (UTR) of CAT-1 mRNA.⁵⁸ In addition, miR-125a represses cell growth in breast cancer cells by targeting HuR.⁶¹ Therefore, HuR seems to be a key factor in the miRNA-mediated regulation of translation of mRNAs containing AREs in their 3' UTR. Whether the functions of HuR in eutopic human endometrium or in explants of endometriosis involve miRNAs remain to be elucidated.

Aberrant posttranscriptional control of ARE-containing mRNAs has been implicated in disease states involving chronic inflammation and cancer.²³ An improved understanding of the ARE-binding proteins that govern the stability and/or translation of ARE-containing mRNA cohorts is anticipated to lead to the development of new therapeutic tools for the treatment of a multitude of inflammatory disorders. Our findings suggest that *HuR* gene expression plays a role in endometrial physiology and possibly in the pathogenesis of endometriosis and need to be validated in further studies with larger sample size. Whether insufficient repression of HuR expression occurs in ectopic endometrial tissues and contributes to the development and growth of endometriotic implants remains to be tested. Whether modulating HuR expression in these cells would constitute a useful therapy for the treatment of endometriosis-related infertility and/or pain also remains to be investigated.

Declaration of Conflicting Interests

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