Coordinate regulation of heterogeneous nuclear ribonucleoprotein dynamics by steroid hormones in the human fallopian tube and endometrium in vivo and in vitro

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MAMMALIAN FALLOPIAN TUBES are a major target of coordinated, synchronized actions of 17β-estradiol (E2) and progesterone (P4) for various tubal functions (24, 35). The fallopian tubes transport gametes for fertilization and initial embryonic development and transport the embryo to the uterus for implantation (24). Our long-standing hypothesis is that E2 and P4 participate in these key physiological processes, which are mediated primarily by transcription factors for estrogen receptors (ERs) and progesterone receptors (PRs) in the fallopian tubes (24). The expression of ER subtypes (ERα and ERβ) and PR isoforms (PRA and PRB) is under dual control of E2 and P4 and is expressed spatiotemporally in human and rodent fallopian tubes (21, 36–38, 41). As master regulators of tubal functions, ERs and PRs govern the expression of downstream target genes. Differential changes in gene expression in fallopian tubal cells are associated with specific stages of the estrous cycle, estrogen-induced tubal transport, development, and endothelin-regulated tubal secretion and contractility (5, 25, 31, 44). Thus, the function of the fallopian tubes appears to involve orchestrated spatiotemporal alterations in transcriptome profiles. Gene expression is regulated at multiple levels (e.g., transcriptional and posttranscriptional) to maintain tubal function under physiological conditions (1). Nonetheless, there remains a need to identify the critical regulators that modulate tissue-specific and RNA-specific expression and the response to steroid hormones in fallopian tubal biology.

Non-specific RNA-binding proteins are important in the posttranscriptional regulation of gene expression in dynamic tissues/cells (7). Heterogeneous nuclear ribonucleoproteins (hnRNPs) are highly conserved, chromatin-associated, RNA-binding proteins that form complexes with RNA polymerase II transcripts and are essential for many cellular processes, including mRNA biogenesis (18, 19). There are about 30 different hnRNPs in humans (7); the most abundant have been characterized and designated hRNP A1 through hnRNP U (18). Most hnRNPs contain multiple RNA-binding domains, which are located generally at the NH2-terminus, bind to sequence-specific elements of DNA/RNA, and interact with other proteins (19). In the nucleus, hnRNPs appear to be functionally diverse. Many hnRNP proteins not only facilitate their processing into mRNAs but also participate in mRNA stability, transport, intracellular localization, and translation by acting as transacting factors (18, 19). In human cells, different hnRNPs have distinct signals that enable them to move between the nucleus and cytoplasm (18).

hnRNPs have received considerable attention because of their roles in regulating transcription and translation in response to steroid hormones and other endocrine mediators (26). Moreover, hnRNP expression contributes to the cellular physiology and development of the endometrium.
The pathogenesis of many diseases (8). The expression of several hnRNP proteins in the uterus has been studied in humans (32), rats (2, 3), and sheep (22, 23); however, the precise roles of hnRNPs during the menstrual cycle and the exact regulatory mechanism that governs their expression in the human fallopian tube and endometrium are unknown.

The identification of different hnRNPs regulated by steroid hormones would provide insight into the molecular control of gene expression in the fallopian tube and the endometrium. We assessed the expression and localization of hnRNPs in human fallopian tubes during the reproductive cycle and correlated the individual hnRNPs with functional end points, such as ovarian expression of steroid hormone receptors (ERα, ERβ1, ERβ2, PRA, and PRB). Because E2 and P4 levels fluctuate during the natural menstrual cycle, we explored the regulatory mechanisms by which steroid hormones facilitate the expression of specific hnRNPs in human endometrial tissues in vitro.

### SUBJECTS AND METHODS

**Sample collection and ethics statement.** Fallopian tube samples were obtained from 17 women undergoing sterilization or hysterectomy (38). The inclusion criteria were regular menstrual cycles (25–29 days), age 28–37 yr (mean: 34.8 yr) with proven fertility (para 1; mean: 2.9), no chronic systemic diseases (e.g., diabetic and inflammatory conditions), and no hormonal therapy for 3 mo before the biopsy collection. Before surgery, all patients were monitored by serial transvaginal ultrasound (Aloka SSD-900/2000; Aloka, Tokyo, Japan) for at least one menstrual cycle (mean: 2 cycles) to determine whether follicles developed. Women received a subcutaneous injection of recombinant human chorionic gonadotropin (rhCG) (250 g, Ovitrelle; Serono International, Geneva, Switzerland) to mimic the natural LH peak when the dominant follicles were 14 and 20 mm (17.1 ± 0.3 mm, means ± SE). The ovulatory stage was determined as described (13); the early phase was defined as 12 to 18 h after rhCG (n = 4), the late phase as 18 to 24 h after rhCG (n = 4), and

### Table 1. Primers: genes, chromosome location, accession no., and source

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<th>Gene</th>
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<td></td>
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hnRNP, heterogenous nuclear ribonucleoproteins; RPLPO, human ribosomal protein. *Human ribosomal protein.

### Table 2. Antibodies: species, clone/catalog no., molecular weight, method, dilution, and source

<table>
<thead>
<tr>
<th>Antibody</th>
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<th>Clone/Category No.</th>
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<th>Method</th>
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<td>1A6</td>
<td>55</td>
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MW, molecular weight; IHC, immunohistochemistry; IF, immunofluorescence; WB, Western blot; ER, estrogen receptor; PR, progesterone receptor; SM, smooth muscle.
the postovulatory phase as ≥24 to 45 h (n = 5) after rhCG. The midsecretory phase (n = 4; cycle days 19–23) was determined from the last menstrual period and endometrial histology in premenopausal women. Immediately before surgery, serum was obtained to confirm the ovulatory and midsecretary phases. The follicle size, hours after rhCG, and E2 and P4 levels for all patients have been reported (38). Laparoscopic surgery for sterilization (3 ovulatory stages) involved clamping and excising the proximal parts (isthmus) of the fallopian tubes, followed by diathermia of the tubal ends (13). The biopsy sample (~0.5 cm) was removed from the abdomen in a laparoscopic sac. All hysterectomy patients were in the midsecretory phase. Hysterectomies were performed for heavy menstrual bleeding in patients who suffered from abdominal pain or vaginal bleeding but had no uterine pathology.

Endometrial tissues from premenopausal (n = 6; aged 28–42 yr, mean 37.2 yr) and postmenopausal (n = 7; aged 63–73 yr, mean 65.3 yr) women were obtained by hysterectomy. The specimens from premenopausal women were classified as late proliferative (LP; cycle days 10–13), midsecretory (MS; cycle days 19–23), and late secretory (LS; cycle days 24–28) phases. The collection and processing steps have been described (39). Two small (3 × 5 × 5 mm) pieces containing the endometrium and the underlying myometrium were excised from the lower uterine cavity. Endometrium was then gently isolated. All tissue samples were washed with ice-cold RNase-free phosphate-buffered saline (PBS) and either snap-frozen in liquid nitrogen and stored at −70°C or fixed in 4% formaldehyde and embedded in paraffin. Endometrial tissues from postmenopausal women who have not taken hormonal replacement therapy for ≥6 mo were used in in vitro experiments. The same biopsy sample was prepared for either qRT-PCR/immunohistochemistry (n = 3) or Western blot analysis/immunohistochemistry (n = 4).

The study was approved by the Research Ethics Committee at Gothenburg University and was conducted at The Sahlgrenska Academy at the University of Gothenburg in accordance with the Declaration of Helsinki for medical research involving human subjects. Informed consent was obtained from all patients.

Human endometrial tissue culture and hormone treatment. After the surgery, endometrial tissues were placed in cold PBS in the operating room and were delivered immediately to the laboratory. After washing in PBS, endometrial tissues were dissected into uniform 0.5- to 1-mm³ pieces with a fine scalpel under a stereomicroscope. The three to five explants were washed three times with RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) and placed in 24-well tissue culture plates (Sarstedt, Newton, MA) containing RPMI-1640 medium with 100 IU/ml penicillin-streptomycin (GIBCO-BRL, San Francisco, CA) supplemented with 10% fetal bovine serum (Sigma-Aldrich), as described previously (6). All procedures were performed within 1 h before the hormonal treatment was started. Serum was stripped with dextran-coated charcoal to remove steroids and other small molecules. The tissues were then treated with human chorionic gonadotropin (hCG) (10 IU/ml; NV Organon, Oss, Holland), E2 (10 nM; Sigma-Aldrich), P4 (100 nM; Sigma-Aldrich), or E2 plus P4 at the same doses as described above, and the cultures were incubated in a humidified incubator (37°C, 95% O2-5% CO2) for 24 h. The selected physiological doses of hCG (29), E2 (21) and P4 (45) were found to ensure effects mediated by LH receptors, ERs, or PR isoforms in human fallopian tubal tissues/cells in vitro. E2 and P4 were dissolved in 100% ethanol at a concentration of 50 mM and added to culture medium to achieve the desired final concentration. Controls were treated with ethanol. Endometrial tissues from each premenopausal woman were treated with vehicle, hCG, E2, P4, or E2 + P4. At the end of the experiments, cultured tissues were snap-frozen in liquid nitrogen and stored at −70°C or fixed in 4% formaldehyde and embedded in paraffin.
Experiments were repeated seven times with a different endometrial donor.

**RNA extraction and quantitative real-time PCR analysis.** Quantitative real-time RT-PCR (qRT-PCR) was performed with an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). The PCR parameters were set according to the manufacturer’s protocols, and the amplification protocol has been described (38, 40). Tissues from the fallopian tubes of each patient were digested with RNase-free DNase I (Ambion) to remove genomic DNA. Total cellular RNA was isolated with the RNeasy Micro Kit (Qiagen) and treated with RNase inhibitor (Applied Biosystems). First-strand cDNA was synthesized from each sample (0.5 μg) with High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). cDNA (1 μl) was added to a reaction master mix (25 μl) containing 1 × SYBR Green PCR reaction mix (Applied Biosystems) and gene-specific primers (500 nM each of forward and reverse primers). For each sample, duplicate reactions were conducted in 96-well plates. All primers were designed to exclude amplification of genomic DNA. TaqMan gene expression products were used for human hnRNPs and human ribosomal protein (Applied Biosystems) (Table 1). Amplification quality was validated by analysis of melting curve. All reactions were performed in duplicate, and each reaction included a nontemplate control. The threshold cycle (CT) values for both RPLPO [human ribosomal protein (NM_001002), provided by Applied Biosystems] and β-actin were not significantly different in any of the groups, which confirmed that the loading was similar between the samples. The results for each gene of interest are expressed as the amount relative to that of RPLPO in each sample. Relative gene expression was determined with the 2−ΔΔCT method (27). We also performed negative control and “no-RT” control reactions.

**Tissue preparation, histology, immunohistochemistry, and immunofluorescence.** For whole-mount examination, fallopian tube and endometrial tissues were washed in cold PBS and fixed overnight in 10% formaldehyde. Paraffin sections (5 μm) were mounted onto slides coated with poly-i-lysine, deparaffinized in xylene, rehydrated through a graded series of ethanol, and stained with hematoxylin and eosin for histological evaluation. Immunohistochemical analysis was performed as described (39). Deparaffinized sections were washed twice for 5 min each in 0.01 M Tris-buffered saline (TBS), pH 7.6. For antigen retrieval, the sections were microwaved for 10 min in 0.01 M citrate buffer (pH 6.0), allowed to cool for 30 min, washed in running water, and blocked in TBS containing 5% normal goat serum (NGS) for 1 h at room temperature. Sections were treated with

Fig. 2. Stage-specific transcription levels of heterogenous nuclear ribonucleoproteins (hnRNPs) in human fallopian tubes during ovulation and in the midsecretory phase. Fallopian tubes from the early, late, and postovulatory phases and the midsecretory phase were analyzed for mRNA levels of hnRNPs (A1, AB, C, D, E, K, H1, G, L, I, and U) by quantitative (q)RT-PCR. The number of tissue samples/group is indicated. The mRNA level of each gene relative to the RPLPO [human ribosomal protein (NM_001002), provided by Applied Biosystems] mRNA level in the same sample is shown. Values are means ± SE. Significance was tested by 2-way ANOVA with Bonferroni correction for multiple comparisons when appropriate. *P < 0.05; **P < 0.01; ***P < 0.001.
Fig. 3. Distribution of hnRNP proteins in human fallopian tubes. Human fallopian tubal tissues from women at different reproductive stages were fixed in formalin and embedded in paraffin. hnRNP expression was determined by immunoperoxidase staining with antibodies to hnRNP A1, AB, D, G, H, and U. Representative images (n = 3–5 patients/group) from 3 independent experiments are shown. A negative immunological control (normal rabbit serum) did not show any staining in the fallopian tubes. The histology of hematoxylin and eosin (H & E)-stained human tubal biopsy samples is indicated. Epi, epithelial cells; str, stromal cells; m, smooth muscle cells. Scale bar, 50 μm.
3% H₂O₂ to remove endogenous peroxidase activity and blocked for nonspecific binding. The primary antibody for all hnRNPs used (Table 2) was diluted 1:1,000 in TBS supplemented with Triton X-100 (TBST) containing 0.05% NGS and incubated overnight at 4°C. After washing with TBST, sections were stained using the avidin-biotin-peroxidase complex detection system (ABC kit; Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Immunostaining was visualized by immersing the sections in 3,3-di-aminobenzidine tetrahydrochloride (0.5 mg/ml in TBS and 0.01% H₂O₂, pH 7.6) for 5 min. Sections were dehydrated and mounted on coverslips with mounting medium (Mountex; Histolab, Gothenburg, Sweden), viewed on an Olympus BX60 microscope (Japan) under bright-field optics, and photomicrographed with the Viewfinder program. To determine the optical density of immunofluorescence staining, we tested various dilutions (1:250, 1:500, and 1:1,000) of hnRNP antibodies in human fallopian tube sections (data not shown). Negative control slides were prepared identically and processed with TBST containing 0.05% NGS, normal mouse IgG, or normal rabbit serum with equivalent concentration in place of primary antibodies. Single and dual immunofluorescence staining were performed as described (38, 41). A cocktail of protease inhibitors was used to decrease tissue degradation and improve the quality of the fluorescent signal. Slides were incubated with antibodies against hnRNPs, ERβ, ERβ2, PRA, and PRB (Table 1) in TBST supplemented with TBST containing 0.05% NGS overnight at 4°C. The optimal working dilutions were based on preliminary experiments at the dilutions reported here. The antibody specificities for ER subtypes and PR isoforms have been reported (10–12, 15, 34) and are shown in Fig. 1. After three 5-min washes in TBST, sections were incubated with Alexa Fluor 488-conjugated goat polyclonal anti-mouse IgG (1:250; Invitrogen, Paisley, UK), Alexa Fluor 594-conjugated goat polyclonal anti-mouse IgG (1:250; Invitrogen), or Alexa Fluor 488-conjugated goat polyclonal anti-rabbit IgG (1:250; Invitrogen) at room temperature for 1 h. Sections were washed and mounted with fluorescent Vectashield with 4',6-diamidino-2-phenylindole (DAPI), viewed on an Axiosvert 200 confocal microscope (Zeiss, Jena, Germany) equipped with a laser-scanning confocal imaging LSM 510 META system (Carl Zeiss), and photomicrographed. Background settings were adjusted on the basis of the examination of the negative control specimens. The controls for nonspecific staining have been described (38, 41). Immunostaining was performed by independent observers who did not know the stage of the cycle or the identity of the protein studied.

Western blot analysis. Whole tissue extracts for protein preparations and Western blot analyses were carried out as described (39, 40). Protein concentrations were determined with the Bradford assay. Laemmli loading buffer (1×) was added to the samples, which were boiled for 10 min and fractionated by gel electrophoresis on 4–12% Bis-Tris gels (Invitrogen). Nonspecific protein binding sites on the polyvinylidifluoride membranes (Amersham International, Buckinghamshire, UK) were blocked by incubating the membrane with 5% nonfat milk in TBS-Tween 20 buffer (10 mM Tris, 150 mM NaCl, and 0.1% Tween 20, pH 8.0) for 4 h. Blots generated with these extracts were probed with primary antibodies (Table 2) overnight at 4°C. The membranes were washed three times for 5 min each in TBS-0.05% Tween 20 and incubated with anti-mouse IgG peroxidase-conjugated goat antibody (A2304; Sigma) or anti-rabbit IgG peroxidase-conjugated goat antibody (A0545, Sigma; 1:5,000 or 1:8,000) for 2 h. Protein bands were visualized with SuperSignal West Dura Extended Duration Substrate (34076; Thermo Scientific, Pierce Biotechnology, Rockford, IL). To reprobe the blot with another antibody, the blot was rehydrated in methanol, rinsed in TBS, and incubated with stripping buffer (46430; Thermo Scientific) for 15 min. Immunoblotted signals were visualized with an LAS 1000 cooled charge-coupled device camera (Fujifilm). Individual bands were quantified directly from membranes by densitometry with Image Gauge software (Fujifilm). Proper loading was evaluated by staining the gels with Coomassie blue. All steps were carried out at room temperature unless otherwise stated.

Coimmunoprecipitation studies. Immunoprecipitation experiments were performed as described (39). Protein extracts (500 µg) were immunoprecipitated with 5 µl of antibody (hnRNP AB, D, G, H, or U) at 4°C overnight. The immunocomplexes were precipitated with 50 µl of Pansorbin cells (Calbiochem, San Diego, CA) for 4 h at room temperature. The bound proteins were washed three times with 1 ml of immunoprecipitation lysis buffer (87787; Thermo Scientific) containing 10 mM iodoacetamide to prevent nonspecific disulfide linkages and twice with 1 ml of PBS. The samples were then analyzed by Western blot to detect hnRNP A1, ERβ, ERβ1, and PR A/B protein expression, as described above.

Statistical analysis. Results are expressed as means ± SE. Significance was tested by one- or two-way ANOVA followed by Bonferroni correction for multiple comparisons as necessary. P < 0.05 was considered significant. Matrices were constructed to calculate the r values (2-tailed bivariate Pearson’s correlation coefficients), and the significance of the r coefficients was calculated on the basis of correlation values and sample sizes. SPSS (version 17.0; SPSS, Chicago, IL) was used for all statistical analyses.

RESULTS

Spatiotemporal expression of hnRNPs in fallopian tubes during the reproductive cycle. In fallopian tube tissues, mRNA levels of hnRNP C, E, I, K, and L did not change during ovulation; however, the levels of hnRNP A1, AB, G, H1, and U mRNA were significantly higher in the late ovulatory phase than in the early ovulatory phase (Fig. 2). hnRNP D expression also differed in the early and postovulatory phases.

To determine the cellular localization of hnRNPs, we performed immunohistochemical analysis of hnRNP A1, AB, D, G, H, and U protein in human fallopian tubes in the late ovulatory and midsecretory phases (Fig. 3). All were localized predominantly to the cell nuclei of epithelial, stromal, and muscle cell layers. In contrast to hnRNP H staining intensity, the immunoreactivities for hnRNP A1, D, and U were higher in

Fig. 4. Physical interaction between hnRNPs and steroid hormone receptors in human fallopian tubes. A: tubal tissue lysates were immunoblotted directly with antibodies against hnRNP A1, AB, D, G, H, or U as indicated. The upper band at 43 kDa for hnRNP A1 may have resulted from posttranslational modifications of hnRNP A1 (e.g., phosphorylation, methylation, or sumoylation). Alternatively, the upper band could signify a different isoform (at least 3 have been identified for hnRNP A1). The cell markers β-tubulin IV, pan-cytokeratin, and α-smooth muscle actin and gels stained with Coomassie blue (data not shown) served as loading controls. Sample 1 was from fallopian tubes in the late ovulatory phase; sample 2 was from fallopian tubes in the midsecretory phase. B: human fallopian tubal sections were immunostained with antibodies to hnRNP A1, hnRNP D, ERα, ERβ1, ERβ2, PRA, or PRB to determine the colocalization of hnRNP A1 (red), ERα (red), ERβ1 (red), ERβ2 (red), or PRA (red) with hnRNP D (green) or PRB (green). The red-green overlay (yellow) shows colocalization of hnRNP A1, hnRNP D, ERα, ERβ1, ERβ2, PRA, and PRB in tubal cell nuclei. Representative images (n = 3 patients) from 2 independent experiments are shown. Scale bars, 50 µm. C: tubal tissue extracts were immunoprecipitated (IP) with hnRNP AB, D, G, H, or U antibody followed by Western blot (WB) analysis with the hnRNP A1 antibody. For comparison, aliquots of whole tubal tissue lysates were analyzed and immunoblotted directly with hnRNP A1. M, molecular weight marker. D: tubal tissue extracts were IP with antibodies to hnRNP AB, D, G, H, or U, followed by WB with ERα, ERβ, or PR antibodies as indicated. Samples 1, 3, 5, 7, 9, and 11 were from fallopian tubes in the late ovulatory phase; samples 2, 4, 6, 8, 10, and 12 were from fallopian tubes in the midsecretary phase.
the late ovulatory phase than in the midsecretory phase. In addition, the intensity of hnRNP AB and G staining did not vary between the late ovulatory and midsecretory phases. As expected, incubating sections with nonimmune IgG from the same fallopian tube tissue did not produce specific immunoreactivity (Fig. 3). These findings were confirmed in immunofluorescence experiments (data not shown).

To further evaluate the expression of hnRNP proteins, lysates of tubal tissue were probed with antibodies against hnRNP A1, AB, D, G, H, and U and analyzed by Western blotting. hnRNP A1, AB, D, G, H, and U were present (Fig. 4A) and corresponded to the predicted hnRNP molecular weights (Table 1). The pattern of changes in the hnRNP A1, AB, D, G, H, and U protein level between the late ovulatory and midsecretory phases paralleled the staining intensity assessed immunohistochemically (Fig. 3). β-Tubulin IV, pan-cytokeratin, and α-smooth muscle actin served as positive controls for the protein content of cilia, epithelial, and smooth muscle cells, respectively (Fig. 4A).

**hnRNPs interact with steroid hormone receptors in fallopian tubes in vivo.** We also examined physical associations between different hnRNPs in the fallopian tubes in vivo. hnRNP A1 and D proteins were concentrated and colocalized in tubal epithelial cells (Fig. 4B). hnRNP A1 also colocalized with AB, G, H, and U (data not shown). Colocalization of hnRNP AB with D, G, H, or U could not be assessed because all of the antibodies were polyclonal rabbit antibodies. Furthermore, hnRNP AB, D, G, H, and U were immunoprecipitated from the tubal tissue lysates, and the immunoprecipitated complex was analyzed for hnRNP A1. Figure 4C supports an interaction between various hnRNPs in the fallopion tubes in vivo.

Previously, we showed that the expression and localization of ER subtypes and PR isoforms are regulated in the fallopion tubes during ovulation and the midsecretory phase (38). Pearson’s correlation analysis revealed that hnRNP A1, AB, D, G, and U mRNA levels correlated with the expression of ER subtype and PR isoform mRNA in a stage-dependent manner (data not shown). Next, we determined whether hnRNPs form complexes with ER subtypes or PR isoforms in the fallopion tube in vivo. hnRNP D colocalized with ERα, ERβ1, ERβ2, and PRA in the nuclei of epithelial and smooth muscle cells (Fig. 4B). hnRNP AB, G, H, and U colocalized with ER subtypes and PR isoforms in nuclei of fallopion tubal cells (data not shown). Interestingly, in anti-hnRNP AB, D, G, H, and U coimmunoprecipitation experiments, hnRNP D, G, H, and U interacted with ERα, whereas hnRNP D and G interacted with ERβ (Fig. 4D). Only hnRNP AB and U interacted with both PRA and PRB. Interestingly, hnRNP G associated specifically with PRA. Moreover, the various hnRNPs appeared to interact with ERs and PRs in the fallopion tubes in a stage-dependent manner (Fig. 4D).

**Coordinate regulation of hnRNPs in the endometrium in vitro.** We hypothesized that the regulation of hnRNP expression might be mediated by LH and steroid hormones. Because of the limited availability of human tubal tissues for the research, we used a tissue culture approach to determine the effect of physiological levels of hCG, E2, and P4 on hnRNP expression in endometrial tissues from postmenopausal women. In the endometrial tissue cultured for 24 h, mRNA levels of hnRNP A1, AB, C, D, E, G, H1, I, K, L or U were not altered by hCG (10 IU/ml), E2 (10 nM), P4 (100 nM), or E2 and P4 (Fig. 5). Although hCG treatment did not significantly alter the hnRNP AB and G mRNA levels, hnRNP AB and G mRNA expression were significantly higher in endometrial tissues treated with E2 than in those treated with hCG. The human endometrium, where ERs and PRs are expressed, is sensitive to cyclical steroid hormone fluctuations in vivo (28). We aimed to determine whether endometrial tissues from postmenopausal women respond to hormone stimulation in vitro. We initially performed immunohistochemistry (Fig. 6A) and Western blot analysis (Fig. 6B) to examine the regulation of ER subtypes and PR isoform protein expression in the endometrium after 24 h in culture. In endometrial tissue cultured with hormones, expression of ERα and PRA/B protein was regulated to a greater extent than in tissue cultured without hormones (Fig. 6, A and B). We also evaluated the effects of hCG, E2, and P4 on the endometrial cell distribution of hnRNP expression by immunohistochemistry (Fig. 7) and Western blot analysis (Fig. 8A). Although the periovulatory LH surge might modulate hnRNP A1, AB, D, G, H1, and U expression in the fallopion tubes in vivo (Figs. 2, 3, and 4A), hCG treatment did not appear to affect the distribution of hnRNP G, H, and U in endometrial tissue in vitro (Figs. 7 and 8A). Immunohistochemical staining revealed that E2 treatment increased the immunoreactivities for hnRNP A1, AB, D, G, H, and U in cell nuclei, especially in epithelial cells (Fig. 7). Western blot analysis confirmed the estrogen-mediated increase in hnRNP A1, AB, D, G, and U protein expression (Fig. 8A). Moreover, P4 had a positive effect on hnRNP A1/U protein levels and a negligible effect on hnRNP AB/G protein levels (Figs. 7 and 8A). Furthermore, the overall hnRNP D and H protein levels in endometrial tissue treated with P4 analyzed by Western blotting were not comparable with their cellular localization detected by immunohistochemical analysis. This divergence might be due to either hnRNP D isoform-specific regulation (Figs. 4A and 8A) (3, 22, 43) or the cell type (epithelial vs. stromal) in the differentiated stages (30). In addition, the combined treatment with E2 and P4 abolished the E2-dependent regulation of hnRNP AB, D, and U expression, whereas combined treatment with E2 and P4 did not affect hnRNP A1, G, and H expression (Figs. 7 and 8A). Moreover, we evaluated cell proliferation in cultured endometrial tissues after the hormone treatments. Treatment with E2 or E2 and P4 together, but not hCG and P4 alone, increased PCNA protein expression in epithelial and stromal cells (Fig. 8B).

**DISCUSSION**

Several lines of evidence suggest that hormone-dependent alterations in gene expression are tightly associated with fallopion tubal (5, 25, 31, 44) and endometrial functions (28). However, the molecular mechanisms of hnRNP expression and regulation through the different stages of the menstrual cycle in the reproductive tissues are not fully understood. This study provides evidence that the mRNA and protein levels of hnRNP A1, AB, D, G, H, and U and the in vivo interactions between hnRNPs in the fallopion tubes are altered during ovulation and in the midsecretory phase. Coimmunolocation and coimmunoprecipitation experiments revealed significant overlap and interactions of hnRNPs and ERs and PRs in the same tubal cell compartments. In cultured endometrial tissue, E2 and P4 directly regulated differential hnRNP expression at the translational level.
Ovarian-derived E2 and P4 are important regulatory factors in the development, differentiation, and maintenance of fallopian tubal functions (24, 35). Our findings on the effects of the menstrual cycle on the regulation of hnRNP A1, AB, D, G, H, and U expression in the fallopian tubes raise an important question: which steroid hormone regulates hnRNP expression? Several studies have shown that steroid hormones can regulate hnRNP expression. For example, Sheflin et al. (43) reported a negative correlation between androgen levels and hnRNP D protein levels in female mice. In addition, in vivo treatment with E2 induces hnRNP D expression in the uterus of ovariectomized rats (2, 3) and sheep (22, 23). This result was in line with the present finding that E2 increased the hnRNP D protein expression in endometrial tissues from postmenopausal women with lower endogenous levels of steroid hormones. Furthermore, E2-stimulated hnRNP D expression was completely blocked by in vivo treatment of rat uteri with ICI-182,780, an ER antagonist (2). In addition, hnRNP E and U interact with ligand-activated androgen receptors (9) and glucocorticoid receptors (14), which affects their transcriptional activities in vitro. These results suggest that specific hnRNP expression and function are regulated in a steroid hormone receptor-dependent and selective manner. Accordingly, in our communolocaton and coimmunoprecipitation experiments, different hnRNPs interacted with specific ER subtypes and/or PR isoforms in fallopian tubes in vivo (Fig. 4, B and D). On the cellular level, the fallopian tubal cell responses to E2 and P4 rely on the expression and activation of ER and PR (21, 36–38, 41). However, our in vitro experiments showed that hnRNP proteins have overlapping but distinct regulation patterns in parallel to trigger ER and PR activation. Although ER- and PR-mediated transcription orchestrate tubal cell functions (35), we do not know how many genes belong to each of the ER or PR targets in the fallopian tube. Since different hnRNPs are hetero-associated with ERs and/or PRs, we may have uncovered an integral role of ERs and PRs in hnRNP regulation, which in turn control physiological homeostasis altered by the actions of the hnRNPs in the fallopian tube. Furthermore, interactions between hnRNP proteins (Fig. 4C) may enable transacting factors to communicate with each other to elicit unique biological responses that cannot be achieved by individual hnRNPs alone.

Changes in hnRNP protein levels were not always accompanied by changes in the corresponding mRNAs in the fallopian tubes in vivo or hormone-treated endometrial tissues in vitro. Although it is not clear whether transcription and translation rates for individual hnRNPs are different, hnRNPs can be regulated quickly at the level of transcription. Translational regulation of hnRNPs is not dependent on mRNA half-lives in response to hormonal stimuli, although it should be stressed that our in vitro experiments used 24-h culture conditions for endometrial tissues treated with different hormones. Therefore, additional studies,
such as time course experiments of hnRNP mRNA and protein profiling in cultured endometrium, may help to refine this picture.

In humans, ~92–94% of genes are alternatively spliced, which results in diverse gene expression patterns in tissues/cells (20). A previous in vitro study demonstrated that E2 does not alter the splicing pattern in the presence or absence of ERα. However, in the absence of PR, P4 failed to change the splicing pattern (4). Thus, steroid hormones may affect the processing of ER subtype and PR isoform proteins in cultured endometrial tissues from postmenopausal women. Human endometrial tissues treated with vehicle, hCG, E2, P4, or E2 + P4 for 24 h were fixed in formalin and embedded in paraffin. ER subtype and PR isoform expression were determined by immunoperoxidase staining with antibodies against ERα, ERβ1, ERβ2, PRA, and PRB. Representative images (n = 2 patients) from 3 independent experiments are shown. Scale bar, 100 μm.
Fig. 7. Distribution of hnRNP proteins in cultured human endometrial tissues. Endometrial biopsy samples from postmenopausal women were treated with vehicle, hCG, E2, P4, or E2 + P4 for 24 h, fixed in formalin, and embedded in paraffin. hnRNP expression was determined by immunoperoxidase staining with antibodies to hnRNP A1, AB, D, G, H, and U. Representative images (n = 3 patients) from 3 independent experiments are shown. The histology of H & E-stained human endometrial biopsy samples is indicated. Scale bar, 100 μm.
of alternative splicing in a steroid receptor-dependent manner. hnRNP proteins are major regulators of the splicing process (18, 20), and our data provide evidence for a physical interaction between hnRNPs and steroid hormone receptors in the fallopian tubes. Thus, hnRNPs and steroid hormone receptors could be intricately connected in the networks that control target gene expression and, in this way, regulate differentiated functions in the fallopian tubes in vivo.

Changes in gene expression are highly coordinated by RNA-binding proteins, microRNAs, and transcriptional factors (16). A number of genes expressed in the fallopian tube are critical for normal tubal function (5, 25, 31, 44). Since hnRNPs regulate mRNA biogenesis (18, 19), hormonal regulation of hnRNPs adds layers of complexity to the gene expression patterns in the fallopian tubes. hnRNP A1, H1, and R can bind to a number of pri-miRNAs and positively or negatively affect the miRNA maturation (17, 47). Our laboratory has demonstrated that Dicer1, which is required for the generation of mature miRNAs, is regulated in human fallopian tubes in a stage-dependent manner. Similar RNA-binding domains are found in both hnRNPs and Dicer1 (16). The data gathered to date suggest that hnRNPs and microRNAs could coordinate to regulate gene expression in fallopian tubes and their specific functions in response to cyclical steroid hormone fluctuation.

We also demonstrated that hCG differentially regulates hnRNP A1, AB, and D expression in the endometrium in vitro. LH receptors are expressed in human fallopian tubes and endometrium, and hormonal regulation of LH receptors may be involved in gamete/embryo transport, fallopian tube contraction, and uterine growth and relaxation (48). Thus, further studies are needed to elucidate the mechanisms of the different regulation of hnRNPs by LH.

In summary, our findings show that hnRNPs are differentially expressed in human fallopian tubes during ovulation and the midsecretory phase and suggest that steroid hormone-dependent regulation of different hnRNPs in the endometrium in vitro reflects different interactions between hnRNPs and/or ERs, PRs, and various hnRNPs functions in vivo. Understanding hnRNP expression during the reproductive cycle will provide insight into the diverse functions of individual hnRNPs and has important implications for elucidating the mechanisms of hnRNP-mediated gene regulation in the fallopian tubes and uterus. Since hnRNP C and U are essential for postimplantation mouse development (33, 46), and since hnRNP A1 protein
levels are increased in the human uterus during pregnancy (32), different hnRNPs may be important in implantation and pregnancy. Of note, the expression of hnRNPs is altered frequently in pathological conditions (8), and dysregulation of hnRNP function results in the progression of multiple diseases (18). Because normal tubal transport is a prerequisite for implantation, retention of gametes or embryos as a result of tubal cell damage may lead to tubal implantation (35, 42). Future studies will focus on examining the significance of the cycle-dependent, steroid hormone-regulated hnRNPs in fallopian tubes from women with ectopic pregnancies and attempt to determine their potential contribution to the development of different tubal diseases.

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