Ciliated epithelial-specific and regional-specific expression and regulation of the estrogen receptor-β2 in the fallopian tubes of immature rats: a possible mechanism for estrogen-mediated transport process in vivo

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Shao R, Weijdegård B, Fernandez-Rodriguez J, Egecioglu E, Zhu C, Andersson N, Thurin-Kjellberg A, Bergh C, Billig H. Ciliated epithelial-specific and regional-specific expression and regulation of the estrogen receptor-β2 in the fallopian tubes of immature rats: a possible mechanism for estrogen-mediated transport process in vivo. Am J Physiol Endocrinol Metab 293: E147–E158, 2007. First published March 20, 2007; doi:10.1152/ajpendo.00101.2007.—Several ER isoforms have been identified in human and rodent tissues, but it is unclear whether each isoform has distinctly different cellular targeting characteristics and physiological functions. We have investigated the intracellular localization and regulatory patterns for ERβ isoforms in rat fallopian tubes. Western blot analysis reveals that two ERβ isoforms corresponding to ERβ1 and ERβ2 are expressed in rat fallopian tubes. However, ERβ2 is the predominant form of ERβ in this tissue. High-resolution confocal imaging and immunohistochemical analysis provide ample evidence that ERβ expression is limited almost exclusively to the ciliated epithelial cells, in contrast to ERα, which is widely distributed. Furthermore, within the ciliated epithelial cells, ERβ is colocalized with β-tubulin IV at stem portion of the cilia. We show that ERβ2 protein expression is tightly regulated by E2 or DPN in a time-dependent manner without changes in ERβ1 expression. These estrogenic effects are inhibited by an ER antagonist, ICI 182,780. In addition, significant alteration of ERβ immunoreactivity is detected only histologically in the ampullary region. Since the cilia are considered an essential determinant of tubal transport, we further demonstrate that E2- or DPN-induced ERβ2 activation is associated with alterations in tubal protein expression crucial for the regulation of calcium-dependent ciliary beating. Given the coordinated regulation and interaction of ER and progesterone receptor in the cilia, we hypothesize that tubal ERβ2 may facilitate the estrogen-mediated transport process by processing protein-protein interaction under physiological and/or pathological conditions. We show for the first time that a previously unrecognized localization of ERβ isoform in rat fallopian tubes can combine with estrogen to individually control the expression of ER β-isoforms in normal target tissues.

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tions of this subtype in mammals is undefined. Previously, sequence analysis has demonstrated a high degree of homology between ERα and ERβ in the DNA-binding domain and the ligand-binding domain (21). Although hormone-activated ERα and ERβ recognize the same estrogen-responsive element (ERE) in the promoter region of target genes, both receptors can display diverse transactivational properties in a ligand-dependent manner when they are coexpressed (54), and ERβ also has the capacity to regulate ERα function in estrogen target cells (22, 57) by altered expression of c-Jun and c-Fos, which can form functional heterodimers for ER-mediated transcription (36). These observations strongly implicate that differential expression ER subtypes are crucial for the induction of tissue/cell-specific responses to various ligands. The importance of ER in female reproduction has been demonstrated using gene knockout models. Not surprisingly, ER subtype-specific knockout mice (15, 28, 30) or mice lacking both ER subtypes (9) show abnormal ovarian and uterine phenotypes. Additional evidence that the fallopian tube appears normal during development but lacks responsiveness to estrogen and is hypoplastic is found in the female ERα/β knockout mice (9). However, one limitation to elucidating the physiological significance of specific ER subtype-mediated effects in the fallopian tubes has been the lack of availability of these knockout mice.

The relationship between hormone receptor-expressing cells and tubal function is a fundamental issue in fallopian tube biology, and earlier studies (43, 44, 60, 61) have demonstrated that specific binding sites for E2 can be observed using the ligand-binding assay in human fallopian tubes. This E2-binding activity is probably due to both ER subtypes. Furthermore, it has been demonstrated (2, 59, 62) that ERs display cyclic changes in human fallopian tubes during the menstrual cycle. Later studies have shown that ER mRNAs and proteins can be detected by RT-PCR (49, 50), in situ hybridization (41, 42, 75), and immunohistochemical analyses (34, 49, 50, 56, 64, 75) in rat fallopian tubes and in particular that ERα expression is also regulated during development and the estrous cycle (41, 50, 75). All of these lines of observations suggest that intracellular ER levels are controlled by estrogen to favor the proper cellular functions in the fallopian tubes. However, the exact nature of expression and cellular localization of ERβ in the fallopian tube remains controversial. In rat fallopian tubes, several studies (56, 75) have detected ERα mRNA and protein in the epithelial and muscle cells where ERβ was absent. Others have reported ERβ mRNA and protein in the epithelial cells (64) of the infundibulum and ampulla regions (50) and in the muscle cells (41, 42, 75). In contrast to ERα in the fallopian tubes, the cell-specific and regional-specific ERβ localization in the fallopian tubes has not been clearly elucidated in most cases. Furthermore, several ERβ isoforms have previously been identified in human and rodent tissues (13, 29, 40). However, the cellular distribution of ERβ isoforms in the fallopian tubes has not been previously reported, and information on the steroid hormones controlling the ERβ isoform expression in the fallopian tubes is lacking.

Therefore, the specific aims of this study were 1) to determine whether the different ERβ protein isoforms are expressed in rat fallopian tubes with the use of a previously characterized antibody to ERβ, 2) to investigate whether ERβ expression is localized to specific regions and cell populations, and 3) to demonstrate how steroid hormones regulate this expression and localization. Furthermore, ciliary motility plays an essential role in fertilization (26), and intracellular calcium (Ca^{2+})-dependent ciliary beat frequency (CBF) (63) of ciliated epithelial cells has been suggested to play an important role in tubal transport in human fallopian tubes (31). The idea that estrogen-regulated ERβ expression may provide this function was supported by analysis of Ca^{2+}-binding proteins in rat fallopian tubes. In addition, the functional ER-mediated effect was evidenced by demonstration of progesterone (P4) receptor (PR), an estrogen-regulated protein, in the fallopian tubes by using the same experimental setting. Our results provided direct evidence of ERβ protein expression in distinct cell populations and new sites for estrogen action in rat fallopian tubes.

**MATERIALS AND METHODS**

**Antibodies**

The primary antibodies used for immunohistochemistry and Western blot analysis in the present study, their dilution, and sources are listed in Table 1. The antibody against ERα was a mouse monoclonal

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**Table 1. Antibodies: species, clone/catalog number, method, dilution, and source**

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<th>Antibody</th>
<th>Species</th>
<th>Clone/Cat. No.</th>
<th>Method</th>
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<td>557035</td>
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ER, estrogen receptor; IHC, immunohistochemistry; WB, Western blot; α-SM, α-smooth muscle; PR, progesterone receptor.
IgG raised against the full length of human ERα (50, 56). The antibody against ERβ was a rabbit polyclonal IgG raised against a synthetic peptide consisting of amino acids 54–71 of rodent ERβ. It recognizes both long form and splice variants of ERβ but does not cross-react with ERα (27, 49). The specificity of these antibodies against the classical nuclear ERα and ERβ have been validated by us in the uterine tissues from wild-type, ERα/β, ERα, and ERβ knockout mice by Western blotting analysis prior to use in this study (data not shown) and by others in previous studies (15, 55). A biotin-conjugated anti-rabbit antibody (711-066-152) streptavidin conjugated with fluorescein (DTAF; 016-010-084) and Cy3-conjugated anti-mouse antibody (715-166-150) was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA) and used for fluorescence immunohistochemistry. Normal mouse IgG and rabbit IgG used as negative controls for immunohistochemistry were purchased from Santa Cruz Biotechnologies. Alkaline phosphatase conjugated goat anti-mouse IgG (A-1682; Sigma) and alkaline phosphatase-conjugated goat anti-rabbit IgG (AC31RL; Tropix, Bedford, MA) were used as secondary antibodies in the Western blot analysis.

**Animals**

All experimental procedures and protocols used in the present study were approved by the local ethics committee, Gothenburg University, Gothenburg, Sweden. Prepuberal female Sprague-Dawley rats were obtained from B&K Universal (Sollentuna, Sweden) and maintained under defined conditions within a temperature-controlled (21 ± 2°C) and light-controlled (12:12-h light-dark cycles) room. They had access to standard pellet food and tap water ad libitum. All animals were allowed to adapt to their new environment for 5 days before studies were initiated.

**Hormonal Treatment and Tissue Preparation**

**Experiment 1.** To ascertain the expression of ER subtypes in female rat reproductive tissues, ovary, fallopian tube, and uterus from 21-day-old female rats (n = 3, 60 ± 5 g body wt) were collected and freed from fat and connective tissue immediately after being killed. Tissues were frozen in liquid nitrogen for Western blot analysis. **Experiment 2.** It has been previously reported that the concentration of E2 is consistently low throughout neonatal development and starts to increase after day 28 of age in rats (45). Therefore, to examine whether acute exogenous estrogen regulated ERβ expression in the fallopian tube, 21-day-old female rats, chosen at random, were given a single intraperitoneal injection of 50 ng/g body wt E2 (Sigma) in 200 μl of vehicle (sesame oil) or vehicle alone. The selected dose of E2 and the treatment regime has previously been shown to be effective in rat uterus in vivo (58).

**Experiment 3.** To further examine whether regulation of ERβ expression by exogenous estrogen is mediated via ERα expression, 21-day-old female rats were given a single intraperitoneal injection of a selective ERβ ligand (37), diarylpropionitrile [DPN; 2,3-bis(4-hydroxyphenyl) propionitrile; Tocris Cookson, Bristol, UK], 100 μg/g body wt, in 200 μl of vehicle (sesame oil) or vehicle alone. We maintained under defined conditions within a temperature-controlled (21 ± 2°C) and light-controlled (12:12-h light-dark cycles) room. They had access to standard pellet food and tap water ad libitum. All animals were allowed to adapt to their new environment for 5 days before studies were initiated.

**Protein Extraction and Western Blot Analysis**

Whole tissue extracts for protein preparations were essentially carried out as described previously (66). Ten millimolars iodoacetamide were included in each buffer used for protein preparations to prevent nonspecific disulfide linkage. One micromolar sodium orthovanadate as a phosphatase inhibitor was also added in the buffer for protein preparation. The protein content was determined using the BCA protein assay (Pierce, Rockford, IL). Immunoblot analyses were performed using standard procedures to evaluate the abundance and distribution of ERα, ERβ, β-tubulin IV, pan-cytokeratin, α-smooth muscle actin, β-actin, e-Jun, c-Fos, PR, calbindin-D9K, and calbindin-D28K (65, 66). Thirty micrograms of protein were directly electrophoresed on 4–12% sodium dodecyl sulfate (SDS)-polyacrylamide gels (Novex, San Diego, CA) using the MOPS-SDS running system. Blots generated with these extracts were probed with primary antibodies. The blots were also probed with an antibody to pan-cytokeratin to examine epithelial content (39) or α-smooth muscle actin to determine smooth muscle content (68). The immunosignal-CDP-Star substrate for alkaline phosphatase (Tropix) was used to visualize protein bands. To reprobe the blot with another antibody, the blot was rehydrated in methanol, rinsed, and incubated with stripping buffer (65 mM Tris·HCl, 2% SDS, and 100 mM β-mercaptoethanol, pH 6.8) at 50°C for 30 min. Immunoblotted signals were visualized using a LAS 1000-cooled charge-coupled device camera (Fujifilm) and ECL film (Amersham). Individual bands were quantified directly from membranes by densitometry using the Image Gauge software (Fujifilm). Signal intensities of the rat ER proteins were normalized to the gels stained with Coomassie blue as ratios to produce arbitrary densitometric units (ADU) of relative abundance. To standardize the assay for measurement of ER proteins, we examined different starting protein concentrations for each sample. This study demonstrated the linearity and validity of ADU for all immunoreactive bands in Western blot analysis (65, 66). All steps were carried out at room temperature unless otherwise stated.

**Immunohistochemical Analyses and Microscope**

Two different methods of immunohistochemistry were performed and confirmed in the same rat fallopian tube tissue. 3,3-Diaminobenzidine tetrahydrochloride (DAB) (65, 66) and dual-fluorescence (67) immunohistochemistry were based on the previously described methodology. After deparaffinization and rehydration, antigen retrieval was completed with 10 mM sodium citrate buffer (pH 6.0, 10 min in a 700-watt microwave). The endogenous peroxidase and nonspecific binding were removed by incubation with 3% H2O2 for 10 min and 10% normal goat serum for 1 h at room temperature. After incubation with primary antibody overnight at 4°C, sections were stained using the avidin-biotinylated-peroxidase complex detection system (ABC kit; Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions, followed by a 2-min treatment with DAB. Sections were imaged on a Nikon E-1000 microscope under bright-field optics and photomicrographed using Easy Image 1 (Bergström Instruments).

For dual-fluorescence immunohistochemistry, tubal sections were blocked in PBS containing 1% BSA-3% fat-free milk for 1 h at room temperature. Slides were incubated with the primary antibody in PBS supplemented with Triton X-100 (PBST) containing 1% BSA-3% fat-free milk overnight at 4°C. After being washed in PBST for 3–5
min, sections were incubated with secondary antibody at room temperature for 1 h. To detect the bound primary antibodies, the secondary antibody, either a biotin-conjugated anti-rabbit antibody together with streptavidin conjugated to DTAf or Cy3-conjugated anti-mouse antibody was employed for different ER subtype staining. Sections were washed and mounted with fluorescent Vectashield with 4,6 diamidino-2-phenylindole (DAPI). Slides were viewed on an Axiovert 200 confocal microscope (Carl Zeiss, Jena, Germany) equipped with a laser-scanning confocal imaging LSM 510 META system (Carl Zeiss) and photomicrographed. Background settings were adjusted from examination of negative control specimens. Images of positive staining were adjusted to make optimal use of the dynamic range of detection. Figures were composed in Adobe Photoshop with minimal alteration for presentation and layout. All final immunohistochemistry was carried out in parallel under identical conditions. To control for nonspecific staining, adjacent sections were stained as above, except the primary antibody was replaced with either PBST, normal mouse IgG, or rabbit IgG 1 in place of both primary antibodies to control for nonspecific staining and to obtain the background level of fluorescence and 2) to replace the second sequence primary antibody to ensure cross-reactivity between the two staining sequences. Rat ovarian and uterine tissues served as a positive control and were stained as above for ER subtypes. The immunohistochemical findings illustrated are representative of those observed in randomly selected sections from multiple animals. The resulting stain was evaluated by two blind observers.

Fig. 1. Expression of estrogen receptor (ER) subtypes and cell type-specific marker proteins in rat reproductive tissues. A: Western blot analysis of ovary, fallopian tube, and uterus from 21-day-old rats. Protein extracts from total lysates (30 μg/lane) were analyzed using antibodies to ERβ, ERα, β-tubulin IV, pan-cytokeratin, α-smooth muscle (α-SM) actin, and β-actin as described in MATERIALS AND METHODS. The molecular weight of the protein is indicated. ERβ1 and ERβ2 (expected size ~59 and 56 kDa) were identified. B: quantification of ER subtype protein expression. The correct loading was evaluated by staining the gels with Coomassie blue. Relative levels of ER subtype proteins were expressed as a ratio of ER densitometric value to whole proteins in Coomassie blue staining. Graphed results are means ± SE of 3 rats/group. ***P < 0.001. C: representative Western blots of tubal protein for ER subtypes and β-tubulin IV. Blot was probed with specific antibody against either ERβ, ERα, or β-tubulin IV as described in MATERIALS AND METHODS, demonstrating specific of the antibody for the ERβ isoforms of appropriate molecular weight in rat fallopian tubes. The apparent molecular weights are shown on the ordinate for 2 standard proteins (ERαs and β-tubulin IV).

Statistical Analysis

Data are means ± SE of the number of independent experiments indicated in the figure legends. Multiple comparisons between data were performed using one-way ANOVA with correction of P values using the Bonferroni’s multiple-range tests under the Analyze-It program (Analyze-It Software). Significance was accepted at the 0.05 level of probability.

RESULTS

Expression of ERβ Isoforms in the Reproductive Tissues in Prepuberal Rats

Known ERβ protein isoforms result from alternative splicing of the ERβ gene, and four ERβ isoforms exist, including the ~59-, 56-, 54-, and 52-kDa ERβ1, ERβ2, ERβ4, and ERβ5 (29). To validate the molecular identity of ER subtypes, we performed Western blot analysis with protein extracts from rat ovary, fallopian tube, and uterus. All extracts exhibited a protein doublet of 51–64 kDa, which was specific for ERβ (Fig. 1A), although the long-form band was substantially larger than the predicted size of ERβ1 (~59 kDa). The antibody to ERβ revealed that ERβ1, considered to be the long form, is the most prominent in the ovary (10, 69). However, the short form of ERβ appeared to be more pronounced in the fallopian tube.
and uterus than in the ovary (Fig. 1, A and B). In support of this notion, a mouse monoclonal anti ERβ antibody (EMR02; Novocastra Laboratories, Newcastle, UK) that did not show cross-reactivity to the other isoforms of ERβ was used to confirm that rat fallopian tubes contained a minor amount of ERβ1 (data not shown), in agreement with a previous study (10). When the membrane proteins from the fallopian tubes were run in the same gel and the blot was cut and probed with either the anti-ERβ, the anti-ERα, or the anti-β-tubulin IV antibody, the immunoreactive band for the ERβ short form migrated slightly slower than β-tubulin IV (55 kDa; Fig. 1C), suggesting that this short form is in close agreement with the molecular mass of ERβ2 (56 kDa) (29). Furthermore, the average relative expression of ERβ1 to ERβ2 proteins in rat ovary (Fig. 1B) was similar to that of their mRNAs in human ovary, as previously reported (29), supporting the concept that the short form of ERβ detected in the present study is ERβ2. Although it remains possible that one additional smaller band in all tissues examined represents proteolytic products generated from the ERβ bands, we do not favor this interpretation, since this smaller band was detected in the uterine tissues isolated from ERαβ and ERβ knockout mice (data not shown). In addition, the expression patterns of ERα and cell type-specific marker proteins are shown for comparison (Fig. 1A). Western blot analysis demonstrated the ERα was most abundant in the fallopian tube and uterus (Fig. 1, A and B). It is important to note that the fallopian tubes expressed both β-tubulin IV [a specific marker for the ciliated epithelial cells in the neonatal and adult fallopian tubes (48, 50)] and pan-cytokeratin, which is widely used for identification of epithelial cells (39). The expression of α-smooth muscle actin protein (68) was included as a positive control for tubal and uterine protein content. Antibody to the ubiquitously expressed β-actin was used to confirm equal loading. In conjunction with the levels of cell type-specific marker proteins detected by Western blot analyses, it can be concluded that ERβ2 protein is dominant in rat fallopian tubes.

Region-Specific Localization of ERβ in Rat Fallopian Tubes

Given that both ERα and ERβ are expressed in the fallopian tubes, we next examined the precise cellular distribution of ER subtypes in each cell compartment of rat fallopian tubes by double-immunofluorescence labeling using a combination of ERα and ERβ antibodies. In all regions examined, the cellular localization of ERα and ERβ was distinguishable. Immunoreactivity of ERα was detected in ciliated and secretory epithelial cells as well as smooth muscle cells (Fig. 2, A–C, left, red), which is consistent with the previous reports on the predominance of ERα in rat fallopian tubes (34, 48–50, 56, 75). However, the positive staining of ERβ was located mainly in the epithelium, placing it close to gametes and or early embryos found in the tubal lumen (Fig. 2, A–C, green). Furthermore, there was a progressive decrease in the proportion of ERβ-positive cells from the infundibulum to the isthmus regions (Fig. 2, A–C), in agreement with the existence of epithelial cell number gradient in the fallopian tubes (1, 26, 31).

Cell Type-Specific Localization of ERβ in Rat Fallopian Tubes

To clearly visualize the exact localization of ERβ in the epithelial cells, we subsequently examined the expression of ERβ in the tubal epithelium using DAB immunostaining. The ciliated epithelial cells were intensely labeled by ERβ antibody (Fig. 3, A and B), which is consistent with Fig. 2, whereas secretory epithelial cells were negative for ERβ (Fig. 3B). Interestingly, intense ERβ immunoreactivity was localized in the stem portion of cilia in the epithelial cells. There was no ERβ immunoreactivity in the cytoplasm and nuclear bodies of ciliated epithelial cells (Fig. 3B). Preincubation of the antibody with the respective immunogen (data not shown) or the control rabbit IgG (Fig. 3C) resulted in no staining, whereas intensive nuclear ERβ immunostaining was detected in rat ovarian granulosa cells when the same antibody was applied (Fig. 3, D and E), thus demonstrating the specificity of the DAB staining in the fallopian tube.

With the goal of further identifying the subcellular compartment containing the majority of ERβ in the ciliated epithelial cells, confocal microscopy was used to compare the distribution of ERβ and β-tubulin IV. Colocalization of ERβ (green) and β-tubulin IV (red) immunoreactivity in the ciliated epithelial cells was clearly visible in the merged pictures (Fig. 3F, yellow). Taken together, these results definitively show that ERβ is localized in the cilia of epithelial cells.

Estrogen Directly Regulates ERβ2 Expression in Rat Fallopian Tubes

Next, we tested the hypothesis that the expression levels of ERβ2 in the ciliated epithelial cells would be altered by acute E2 stimulation. Following treatment of prepuberal rats with E2 (0–24 h), ERβ2 levels gradually increased, whereas additional treatment with ICI 182,780 slowly lowered ERβ2 levels, consistent with its known ER antagonist activity (25). This inhibitory effect was shown to be time dependent (Fig. 4A). In contrast, ERβ1 expression remained at almost background levels in all tubal extracts, and there were virtually no corresponding changes in ERβ1 expression during the same time treatment period (data not shown). Therefore, most of the cell-specific immunoreactivity corresponding to ERβ reflects ERβ2 activity in the cilia.

To further test the specificity of the estrogenic effect on ERβ2 expression, DPN, a selective ERβ ligand (37), was used to mimic the action of E2 that directly regulates and activates ERβ. DPN induced ERβ2 expression in a similar fashion as E2, and expression was also inhibited by ICI 182,780 (Fig. 4B). There was no corresponding increase in ERα expression during DPN treatment, although a significant reduction in ERα levels was observed after addition of ICI 182,780 (Fig. 4B). In contrast, no changes in either ER subtype were observed in the fallopian tubes from rats treated with vehicle alone (sesame oil; Fig. 4C). In addition, the specificity of the E2 effect in the fallopian tubes was determined by testing 17α-estradiol (50 ng/g body wt in 200 μl of sesame oil) that does not activate the ER subtypes (data not shown). These results suggest that induction of ERβ2 expression by E2 treatment is unlikely to be mediated via increases in ERα levels in rat fallopian tubes.

To examine whether the expression of ERβ2 increases differentially in different tubal regions, we analyzed ERβ2-
positive epithelial cells in the tubal epithelium following E2 and/or ICI 182,780 treatment by immunohistochemistry. Consistent with the Western blot data (Fig. 4A), immunoreactive ERβ increased in the ampullary region with E2 treatment (Fig. 4E) and was reduced after addition of ICI 182,780 (Fig. 4F). No change was observed in the infundibulum (data not shown) and isthmus (C) regions. Whereas ERα was ubiquitously expressed in all cell types without regional difference in the fallopian tubes, ERβ was detected mainly in the tubal epithelium, with the most intense signals in the infundibulum region (A), and detected less in the isthmus region (C). Sections were subsequently counterstained with DAPI to visualize cell nuclei (A–C, right). Using tubal tissues from different rats, the same results were obtained for replicate experiments. Scale bars are shown. epi, Epithelial cells; m, smooth muscle cells; lu, lumen.

Effects of Estrogen on ERβ-Associated Signaling Proteins in Rat Fallopian Tubes

The functional effects of estrogen occur through activation of ERs and modulation of gene expression, one of the events probably initiated by binding to AP-1, which is composed of Jun and Fos proteins (21, 36). Since most AP-1 transcription factors are present at low levels in cells but are rapidly induced and activated in response to specific stimuli, we measured the “activational” expression of c-Jun and c-Fos protein in the fallopian tubes from rats treated with E2, DPN, or ICI 182,780. c-Jun protein was abundantly increased after 0.5 and 1 h of E2 treatment. In addition, the level of c-Jun protein appeared to be increased by DPN in a time-dependent fashion. The amounts of c-Jun were not largely altered after ICI 182,780 treatment in E2-primed rats. In contrast, treatment with ICI 182,780 markedly decreased c-Jun protein levels in DPN-primed rats (Fig. 5, A and B), suggesting that the regulation of c-Jun may be an ERβ-related manner. On the other hand, little or no change in the expression levels of c-Fos was observed after either E2, DPN, or ICI 182,780 exposure (Fig. 5, A and B). The results of this analysis demonstrate that changes in ER subtype protein
levels by E₂, DPN, or ICI 182,780 occurred independently of c-Fos status.

To answer the question of whether estrogen exposure could affect the sensitivity of estrogen-regulated protein expression, rats were treated at different times in the presence of E₂ and/or ICI 182,780 and PR, and calbindin-D9K and calbindin-D28K were examined. Western blot analysis revealed that E₂ was able to induce PR(A/B) and calbindin-D9K expression in a time-dependent manner. This E₂ effect was reversed by ICI 182,780, suggesting that both PR(A/B) and calbindin-D9K proteins are involved in E₂-mediated ER activation (Fig. 5A). A slight decrease in calbindin-D28K protein could be detected at 3 h after E₂ treatment. The expression of this protein increased thereafter, becoming highly expressed at a time when high levels of ERβ2 were detected in rat fallopian tubes (Fig. 4A). However, there was no significant change in calbindin-D28K expression during ICI 182,780 treatment (Fig. 5A).

Confirmation of the role of ERβ in the regulation of PR, calbindin-D9K, and calbindin-D28K expression was obtained by DPN treatment. Similarly to E₂, DPN was also able to increase PR(A/B), but with a smaller magnitude compared with E₂ treatment. DPN also increased calbindin-D9K protein levels in a time-dependent manner. Blockage of E₂ effects on PR(A/B) expression was observed by ICI 182,780 treatment (Fig. 5B). Interestingly, when we examined calbindin-D28K protein levels in the same tissue extracts, a decrease in the expression of this protein with time of DPN treatment was observed. The minimum expression of calbindin-D28K was detected after 12-h E₂ treatment, and the level of this protein increased thereafter in a manner similar to E₂ treatment. Furthermore, ICI 182,780 exhibited similar effects to DPN on calbindin-D28K expression (Fig. 5B). There results support the hypothesis linking the regulation of ERβ expression to the functions of Ca²⁺-regulated processes.

\[ P_4 \] Fails to Regulate ERβ2 Expression in Rat Fallopian Tubes

P₄ is often considered as an antagonist in modulating the effects of estrogen in the fallopian tubes (26, 66). It is possible that the response to estrogen is not specific, and P₄ could also regulate ERβ2 expression. To address this possibility, a similar time course experiment was performed to evaluate whether P₄ participates in the regulation of ERβ2 protein expression in rat fallopian tubes. Western blot analysis showed that levels of ERβ2 remained unchanged, whereas ERα protein tended to decrease during P₄ treatment (Fig. 6). Moreover, there were no significant differences in serum P₄ concentrations among rats given oil, E₂, DPN, and/or ICI 182,780 during the treatment period (data not shown), suggesting that regulation of ERβ2 expression is unlikely to be related to P₄ action.

DISCUSSION

Interest in the functions of ERβ has increased due to not only the distinct tissue/cell distribution and opposite transcriptional directions compared with ERα (10, 29, 54, 69) but also due to the discovery of multiple isoforms of this receptor in a variety of tissues/cell types in humans and rodents (13, 29, 40). Furthermore, most experiments on the involvement of ERβ in various cellular (22, 36, 57) and physiological (15, 28) functions have dealt only with the full-length ERβ1, leaving the

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**Fig. 3.** Identification of ERβ localization in the ciliated epithelial cells. Tubal sections were immunolabeled for ERβ visualized with diaminobenzidine (DAB; brown) staining as described in MATERIALS AND METHODS. Antibodies used in Fig. 3. Identification of ER

\[ \text{ERβ} \]
tory epithelial cells lacked specific ERβ immunoreactivity. A: strong ERβ immunoreactivity was selectively restricted to ciliated epithelial cells (secretory epithelial cells lacked specific ERβ immunoreactivity). A zoom of the epithelial cells positive for ERβ in B is indicated by a red solid arrow. C: the same concentration of rabbit IgG instead of the primary antibody and secondary antibody was used as a negative control in an adjacent tubal section. D: in the same experiment, intensive nuclear ERβ immunoreactivity was detected in rat ovarian granulosa cells. E: an enhanced magnification, noted by a black solid arrow in D, indicated an ovarian follicle. Using tubal tissues from different rats, the same results were obtained for replicate experiments. Scale bars are shown. CEC, ciliated epithelial cell; SEC, secretory epithelial cell; GC, granulosa cell; TC, theca cell. F: confocal microscopy of dual-fluorescent staining of ERβ and β-tubulin IV in epithelial cells of rat fallopian tubes. Tubal sections were immunolabeled for ERβ and β-tubulin IV visualized with the appropriate wavelength for ERβ (green), β-tubulin IV (red), and merged (yellow) as described in MATERIALS AND METHODS. Figure shows the high degree of overlap coefficient of ERβ and β-tubulin IV.
The functional significance of other ERβ isoforms elusive. Using several molecular approaches, the present study offers a detailed description of the expression, cellular localization, and regulation of ERβ isoforms within the rat fallopian tubes in response to estrogen stimulation.

Using analysis of protein expression patterns and cell type-specific markers, we observe that ERβ2 is the predominant ERβ isoform and that ERβ1 expression is very low in rat fallopian tubes. The ratio of the two ERβ isoforms has been known to vary depending on the tissue (29, 40), but such an
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exclusive expression of either isoform as seen in the fallopian tubes in vivo has not been shown to date. Strikingly, our study clearly shows that the expression of ERβ is intense in ciliated but not secretory epithelial cells by demonstrating the colocalization of ERβ and β-tubulin IV, a cellular marker for the cilia in the fallopian tubes (48, 50). Similarly, immunoreactivity for ciliated ERβ is also found in human and mouse (Shao R, Wejdegård B, Fernandez-Rodriguez J, and Billig H, unpublished data) fallopian tube epithelium. However, previous studies investigating ER expression in rat fallopian tubes have found either no ERβ protein expression (48, 56) or selective localization in the smooth muscle cells (64). The reasons for divergent results are likely to relate to differences in ERβ antibodies (the differential epitope and antigen recognition), experimental techniques (no discrimination between ERβ isoforms), and/or the phase of the cycle of the animals used in the studies. Also, the failure to observe ERβ in the past is probably due to the extremely low levels of ERβ1, originally described as ERβ (Fig. 1A) (10, 64), in rat fallopian tubes.

To our knowledge, this is the first study to demonstrate that ERβ2 protein but not ERβ1 protein in rat fallopian tubes responds dynamically to estrogen stimulation by undergoing a time-dependent regulation in vivo. As a complementary approach, DPN, an ERβ agonist, was used to test whether estrogen regulates ERβ2 expression directly, since ERα is also present in the same tissues, suggesting that ERβ2 itself may play a role in the modulation of estrogentic action in the fallopian tubes. Moreover, biochemical studies indicate that ERβ2 encodes most functional domains related to ERβ1 (13).

Fig. 5. Western blot analysis of ER-associated signaling proteins in rat fallopian tubes. Representative Western blots of tubal proteins for c-Jun, c-Fos, progesterone (P₄) receptor (PR), calbindin-D9K, and calbindin-D28K protein expression. All extracts were prepared at the time intervals indicated. Blot was probed with specific antibody against either c-Jun, c-Fos, PR, calbindin-D9K, or calbindin-D28K as described in MATERIALS AND METHODS. Antibody to the ubiquitously expressed β-actin was used to confirm equal loading. The data are representative of 2 separate experiments.

Fig. 6. Time course effect of P₄ on ERβ2 protein levels. Representative Western blots of tubal protein for ER subtypes. All extracts were prepared at the time intervals indicated. Blot was probed with specific antibody against either ERβ or ERα as described in MATERIALS AND METHODS. The proper loading was evaluated by staining the gels with Coomassie blue. Relative levels of ERβ2 proteins were expressed as a ratio of ERβ2 densitometric value to whole proteins in Coomassie blue staining. Graphed results are means ± SE of 3 rats/group.
Ca\(^{2+}\)-regulated processes are reflected in the variety of Ca\(^{2+}\)-binding proteins (32). Experimental studies performed in vivo have shown that calbindin-D9K is localized in the ciliated epithelial cells (35) and that it is regulated by estrogen in rat fallopian tubes (7). In addition, the dependency of calbindin-D28K expression on estrogen has been reported in mouse fallopian tubes (19). The time course studies show that the upregulation of ER\(\beta\)2 expression by both E\(_2\) and DPN coincides with an increase in the amounts of calbindin-D9K, whereas calbindin-D28K is transiently decreased in rat fallopian tubes. These correlative findings support the notion that estrogen-induced Ca\(^{2+}\)-binding protein changes may be in part responsible for ER\(\beta\)2 expression and activation, which implicates ER\(\beta\)2 in coordinating Ca\(^{2+}\)-related cilia functions such as ciliary beat. The ability of steroid hormones to regulate CBF has been demonstrated in vitro. For instance, treatment with E\(_2\) prevents P\(_4\)-induced inhibition of CBF in human fallopian tubes, although E\(_2\) itself has no effect on CBF (33), suggesting that estrogen may promote cilia to change CBF indirectly.

Although enhancement of ovum transport by exogenous E\(_2\) is observed in cyclic and pregnant rats (52), any mechanistic explanation of how estrogen regulates CBF in the ciliated epithelial cells and influence tubal transport process (20) is necessarily speculative. We and others have previously shown that in the fallopian tubes gonadotropins increase the level of PRs, which are required for endogenous E\(_2\) stimulation of steroidogenesis in vivo (66, 71) and in vitro (53). In this study, we demonstrate that treatment with E\(_2\) or DPN results in the induction of PR proteins in rat fallopian tubes, further strengthening the notion that estrogen is a positive regulator of PR in rodent fallopian tubes. Both ER and PR are members of the steroid/nuclear receptor superfamily with similar functional domains for ligand binding, nuclear location, transcriptional activation, and DNA binding (72). A previous study has demonstrated that ER is able to interact with the PR at the AP-1 response element (73), and data suggest that the potential exists for cross-talk between ER and PR respond to the integration of the steroid hormone signals (3). More recently, it has been reported that PR can be detected in the cilia of ciliated epithelial cells in human and mouse fallopian tubes (71), which is consistent with the fact that ER\(\beta\) is localized in the cilia (Fig. 3). In line with these observations, it is likely that an ER\(\beta\) and PR interaction is also present in the cilia in rat fallopian tubes.

Since P\(_4\) is not able to regulate ER\(\beta\)2 expression in vivo, we hypothesize that ER\(\beta\)2 acts on cilia to regulate CBF via PR, which may explain the indirect effect of estrogen on the regulation of CBF during transport process in human fallopian tubes. Obviously, our data together with a previous study showing that local exposure to ICI 182,780 in the fallopian tubes blocks E\(_2\)-induced ovum transport in cyclic rats (51) lend strength to the physiological relevance of ER\(\beta\) in this tissue in vivo.

The elucidation of the cellular functions of ER\(\beta\) proteins in rat-ciliated epithelial cells will allow us to reveal the connection between cell type-specific ER\(\beta\) contribution and disease-related tubal dysfunction. In vivo and in vitro observations (8, 14) have previously shown that the number of ciliated epithelial cells is increased by E\(_2\) stimulation in human fallopion tubes. Notably, human tubal ectopic pregnancy shows a reduction in the number of ciliated epithelial cells (6) and low levels of circulating estrogen (4, 46). Beneficial effects of E\(_2\) supplementation have been found in women with tubal ecopic pregnancy (18). These observations indicate that estrogen may determine the number of ciliated cells and coordinate ciliary motility in the fallopion tubes. Results from previous studies (11, 20) also suggest that tubal cilia in the ampullary region rather than other regions are crucial for gamete transport function. Although ciliated-ER\(\beta\) protein is ubiquitously expressed in all tubal regions in rats, our results show that the significant alteration of ER\(\beta\)2 protein expression is observed mostly in the ampullary region. Treatment with ICI 182,780 reduces ER\(\beta\)2 expression in ampullary region cilia but not in other regions, which is in agreement with the finding that the ampulla is the main region affected by reduced number of ciliated epithelial cells when estrogen is abolished (1). Our data here point to a possible clinical relationship. Since fertilization occurs in ampullary region (26, 31), which is also the most common site for tubal ectopic pregnancies in humans (38, 70), it can be assumed that a decrease in ER\(\beta\)2 protein level may be, at least in part, a direct consequence of cilia loss (i.e., deciliation) (8, 14, 26) rather than ciliated epithelial cell death (in this study). Alternatively, lack of estrogen-mediated ER\(\beta\)2 function may result in the occurrence of tubal ectopic pregnancies and reduced fertility. Further studies will be needed to verify this concept and extend it to human fallopian tube abnormality.

In the mammalian fallopian tube, the process of successful fertilization is regulated by a complex interplay of endocrine and paracrine signals, including estrogens (26) that are mediated mostly by nuclear ER-dependent mechanisms via at least two distinct receptors, ER\(\alpha\) and ER\(\beta\) (21). The identification of an ER subtype with restricted expression in specific cell populations yields insights into the physiological functions of the fallopian tubes and provides the basis for potential functional manipulations. Our results demonstrate that ER\(\alpha\) is located in the epithelial and smooth muscles cells, whereas expression of ER\(\beta\) is detected only in the epithelial cells. The differential localization of ER\(\alpha\) and ER\(\beta\) among populations of tubal cells may explain why estrogen affects target cell and respective cellular components differently. For example, E\(_2\) participates in the regulation of smooth muscle contraction and relaxation in rat fallopian tubes (51), suggesting that ER\(\alpha\)-positive smooth muscle cells respond directly to E\(_2\) action. On the other hand, emerging evidence indicates that ER antagonist ICI 182,780 stimulates degradation of ERs and prevents receptor dimerization, inhibiting estrogen-dependent gene transcription (12, 16). In accordance with this, we observe that E\(_2\)-induced ER\(\alpha\) and ER\(\beta\)2 expression is inhibited efficiently by ICI 182,780. However, ER\(\beta\)-positive epithelial cells also contain ER\(\alpha\); therefore, it is not possible to dissect the individual role of ER subtypes in the fallopian tubes in our studies since ICI 182,780 usually results in an attenuated cellular response of E\(_2\) and transcriptional repression of both ER\(\alpha\) and ER\(\beta\) regardless of the presence or absence of ER agonist (25). An interesting area for future investigations would be to enlarge our approach to select ER subtypes on the basis of previously established ER knockout mouse models (9, 15, 28, 30). Further studies to confirm the absence of individual ER subtype in the fallopion tubes will be informative in evaluating their specific function and comparing them with those observed in rat fallopian tubes in vivo.
In summary, we demonstrate for the first time a specific localization and possible function of ERβ isoforms in rat fallopian tubes. We show that ERβ2 represents a relatively high proportion of total ERβ protein. Considering the cellular distribution and regulatory patterns of ERβ2 in ciliated epithelial cells, we speculate that this isoform may function in female reproductive events within the fallopian tube (transport of the female and male gametes as well as embryos) and also when these events are dysfunctional (i.e., induction of tubal ectopic pregnancy). Furthermore, ERβ2 apparently contributes to the action of E2 by changing ciliated epithelial cell function. In this context, the present work not only enhances our knowledge on tissue/cell-specific expression of ERβ isoforms but also provides a physiological link between ERβ2 and tubal transport processes affected by E2 signaling. Future challenges include establishing exactly how ERβ2 is targeted to cilia, what other proteins it interacts with, and what the downstream target of ERβ2 may be. These are under investigation in our laboratory.

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