The estrogen-responsive adrenomedullin and receptor-modifying protein 3 gene identified by DNA microarray analysis are directly regulated by estrogen receptor

H Watanabe, E Takahashi, M Kobayashi, M Goto, A Krust[1], P Chambon[1] and T Iguchi

Okazaki Institute for Integrative Bioscience, National Institutes of Natural Science, 5-1 Higashiyama, Myodaiji, Okazaki 444-8787, Japan and Core Research for Evolution Science and Technology (CREST), Japan Science and Technology Corporation

[1]Institut de Génétique et de Biologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, Université Louis Pasteur, Collège de France, 67404 Illkirch, Strasbourg, France

(Requests for offprints should be addressed to T Iguchi; Email: taisen@nibb.ac.jp)

Abstract

Recent studies have revealed that hundreds of genes in the uterus are activated by estrogen. Their expression profiles differ over time and doses and it is not clear whether all these genes are directly regulated by estrogen via the estrogen receptor. To select the genes that may be regulated by estrogen, we treated mice with several doses of estrogen and searched for those genes whose dose–response expression pattern mirrored the uterine growth pattern. Among those genes, we found the dose-dependent expression of the adrenomedullin (ADM) gene correlated well with the uterotrophic effect of estrogen. ADM expression is induced early after estrogen administration and is restricted to the endometrial stroma. The spatiotemporal gene expression pattern of ADM was similar to that of receptor-modifying protein 3 (RAMP3). RAMP3 is known to modify calcitonin gene-related receptor (CRLR) so that it can then serve as an ADM receptor. Chromatin immunoprecipitation assays indicated that the estrogen receptor binds directly to the ADM promoter region and RAMP3 intron after estrogen administration. It was also shown that neither the ADM nor RAMP3 gene could be activated in estrogen receptor-α-null mouse. Although uterine ADM expression has been reported to occur in the myometrium, our observations indicate that estrogen-induced ADM is also expressed in the uterine stroma and that such variable, spatiotemporally regulated ADM expression contributes to a wider range of biological effects than previously expected.

Journal of Molecular Endocrinology (2006) **36,** 000–000

Introduction

The uterus is a major target organ of estrogen and undergoes drastic changes after estrogen administration. Early uterine responses to estrogen include many physiological biochemical changes such as hyperemia, calcium influx, histamine release, eosinophil infiltration, cAMP level changes, enhanced glucose oxidation, and increased RNA and protein synthesis. After DNA synthesis and mitosis, later stage effects include cellular hypertrophy and hyperplasia and the result is the growth of the uterus. This uterotrophic effect of estrogen is a demonstrable phenotype that can be used to estimate the potency of estrogens. Notably, not all the physiological and biochemical changes associated with estrogen treatment are necessary for the uterotrophic effect. For example, while cAMP is elevated after estrogen administration (Szego & Davis 1967), uterine growth is independent of cAMP (Zor *et al.* 1973). This is also true for ornithine decarboxylation and prostaglandins. Thus, it remains unclear which processes are essential for the effects of estrogen in target tissues.

In the level of gene expression, it is also not clear whether estrogen-response genes are directly regulated by estrogen receptor (ER) or not. Recently, the effect of estrogen on the uterus has been studied at the level of gene expression by DNA microarray analysis (Watanabe *et al.* 2002). As a result, hundreds of genes have been listed as being estrogen-responsive and the temporal changes in their expression after estrogen exposure have been analyzed (Watanabe *et al.* 2003). This has vastly aided our understanding of the effect of estrogen on the uterus at the genetic level. However, this new methodology, like the classical methodology, cannot determine whether a particular gene-expression change is the direct effect of estrogen or not.

In our previous study we found that not all estrogen-responsive genes are uniformly activated by estrogen and that their estrogen-dose-dependent, gene-expression patterns are not necessary identical with the uterotrophic effect of estrogen (Watanabe *et al.* 2003). For example, while the uterotrophic effect of estrogen is directly proportional to the estrogen doses used, some genes are

0952–5041/06/036–000

DOI: 10.1677/jme.1.01825
Online version via http://www.endocrinology-journals.org

activated by a low dose of estrogen but this activation is saturated or diminished by higher doses of estrogen. These various estrogen-response patterns are generally attributed to the differences of promoter context but the contribution of ER is not clear. In this study, we selected the adrenomedullin (ADM) and receptor-modifying protein 3 (RAMP3) genes, whose expression patterns correlate closely with the uterotrophic effect, and studied the direct contribution of ER to their expression.

Experimental procedures

Animals

Female C57BL/6J mice and ERα- and -β-null mice (Dupont *et al.* 2000) were housed under a 12 h:12 h light/dark cycle. To assess the effect of estrogen on uterine gene expression, mice were ovariectomized at 8 weeks of age and 2 weeks later injected intraperitoneally with estrogen (or sesame oil as a vehicle control; Nakarai Tesque, Kyoto, Japan). The whole uteri ($n=4$) were collected 6 h later. The estrogen used was 17β-estradiol (Sigma-Aldrich Japan, Tokyo, Japan) and it was injected at 0·05, 0·5, 5 or 50 µg/kg body weight (b.w.). To assess the effects of the different estrogen doses on uterine growth, mice were ovariectomized and 2 weeks later injected intraperitoneally every 24 h for 7 days with the different 17β-estradiol doses or sesame oil. The whole uteri ($n=5$) were then collected and weighed. All animal experiments were approved by the institutional animal care committee.

Preparation of labeled cRNA and microarray analysis

Total uterine RNA was extracted using TRIzol reagent (Invitrogen, Tokyo, Japan) and cRNA probes were prepared from the purified RNA by using an Affymetrix cRNA probe kit according to the manufacturer's protocol (Affymetrix Japan, Tokyo, Japan). All preparations met the recommended criteria of Affymetrix for use on their expression arrays. The amplified cRNA was hybridized to high-density oligonucleotide arrays (Mouse U74 A; Affymetrix Japan), and the scanned data were analyzed with GeneChip software (Affymetrix Japan) and processed as described previously (Watanabe *et al.* 2002). To confirm the estrogen-related changes in gene expression revealed by the DNA microarray analysis, we independently repeated the same experiment at least twice. The expression data were analyzed with GeneSpring software (Agilent Tech. Japan, Tokyo, Japan).

Quantitative real-time PCR

cDNA was synthesized from total RNA purified as described above by using Superscript II reverse transciptase (-;; Invitrogen) with random primers at **Q1** 42 °C for 60 min. Quantitative PCR reactions were performed by using the PE Prism 7000 sequence detector (Applied Biosystems, Tokyo, Japan), SYBR-Green PCR core reagents (Applied Biosystems) and the appropriate primers according to the manufacturer's instructions. The primers were chosen to amplify short PCR products (< 100 bp), and their sequences were as follows: RAMP1 (NM_016894), TTTCATTGCGCTC **Q2** CCCATT and CCAGACCACCAGTGCAGTCAT; RAMP2 (NM_019444), AGTTGCATGGACTCTGTC AAGGA and TGCCTGCTAATCAAAGTCCAGTT; RAMP3 (NM_019511), CCGGATGAAGTACTCAT CCCA and CCACCAGGCCAGCCATAG; ADM (U77630) ATAAGCCTCATTACTACTTGAACT and TTGCACGTTCCTCGCTAGGT, and CRLR (calcitonin gene-related receptor; NM_018782) CACTCTG ATGCTCTCCGCAGT and GGCTGTACCCTTGC ATGTCAC. Gene-expression levels were normalized to the expression levels of NM_012053 (ribosome L8, Rpl8), whose primer sequences were ACAGAGCCGT TGTTGGTGTTG and CAGCAGTTCCTCTTTGC CTTGT. Gel electrophoresis and melting-curve analyses were performed to confirm correct amplicon size and the absence of nonspecific bands.

In situ hybridization

In situ hybridization was performed using PCR fragments containing a portion of the ADM gene (nucleotides 58–586; U77630), the RAMP2 gene (nucleotides 160–706; NM_019444) or the RAMP3 gene (nucleotides 681–1059; NM_019511). [α-^{35}S]UTP (Amersham Biosciences, Tokyo, Japan)-labeled antisense and sense RNAs were obtained by using T7 and T3 RNA polymerases and an *in vitro* transcription kit (Stratagene, Funakoshi, Tokyo, Japan). After DNase digestion, the probes were fragmented by alkali hydrolysis. The tissues were embedded in OCT and **Q3** sectioned (10 µm) using a cryostat. Sections were fixed on slides with 4% paraformaldehyde for 10 min and rinsed in PBS. The slides were then acetylated in 0·1 M triethanolamine with a 1/400 vol. acetic anhydride, rinsed again in PBS, and dehydrated in graded ethanol. After air drying, the hybridization mixture was added. Hybridization was performed at 50 °C overnight with 5 × 10^7 d.p.m./ml probe in 100 µl hybridization solution under coverslips. After the hybridization, the slides were washed and incubated in RNase A solution (20 µg/ml) at 37 °C for 30 min, and then dipped in NTB-2 nuclear track emulsion (Kodak, Rochester, NY, USA). After 14 days exposure, the slides were developed and counterstained with hematoxylin. The sections were evaluated and photographed under dark-field illumination using a Zeiss microscope. The dark-field (changed to red) and bright-field images were merged.

Chromatin immunoprecipitation

Mouse uteri were fixed with 1% formaldehyde and homogenized using physcotron (NS-310E; Microtec, Chiba, Japan) in PBS containing 0·125 M glycine. The samples were then centrifuged at 700 ***g*** for 5 min at 4 °C and the pellets were incubated with lysis buffer (10 mM Tris/HCl, pH 8·0, 10 mM EDTA, 0·5 mM EGTA and 0·25% Triton X-100) for 10 min. The samples were collected by microcentrifugation, suspended in sonication buffer (10 mM Tris/HCl, pH 8·0, 100 mM NaCl, 1 mM EDTA and 0·5 mM EGTA), and sonicated with a Bioruptor sonicator (Cosmo Bio, Tokyo, Japan) to an average length of approximately 500 bp. The samples were precleared by treatment with Protein G–Sepharose for 1 h at 4 °C and then incubated with 10 μg anti-ERα polyclonal rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-acetylated histone H3 **Q4** antibody (Cell Signaling, MA, USA) overnight at 4 °C. After precipitation by the addition of Protein G–Sepharose the samples were washed five times with RIPA buffer (10 mM Tris/HCl, pH 8·0, 140 mM NaCl, 1 mM EDTA, 0·5 mM EGTA, 1% Triton X-100, 0·1% SDS and 0·1% sodium deoxycholate). The precipitated samples were recovered by incubation with elution buffer (0·1 M sodium bicarbonate and 1% SDS). Crosslinks were reversed by incubation at 65 °C for 6 h followed by incubation with proteinase K at 45 °C for 4 h. Thereafter, the samples were extracted with phenol/chloroform and the DNA fragments were precipitated with a 1/10 vol. of NaCl and 2·5 vol. of ethanol. Generally, 1/30 of the precipitated DNA was used for PCR amplification. PCR amplification was performed in the presence of 0·1 nmol primers, 0·2 mM each nucleotide (dATP, dCTP, dGTP and dTTP), 1 × PCR buffer and 1 U AmpliTaq Gold (PerkinElmer Japan, Tokyo, Japan) in 20 μl of reaction buffer. After 35 cycles of amplification the amplified DNA was analyzed by agarose electrophoresis. As a negative control the same experiments were performed with IgG: no amplified DNA was obtained. The primer sequences used to amplify the putative estrogen-response element (ERE) of the ADM gene were (− 796) 5′-ATCCTCA CGTTTATGATGGA-3′ (− 777) and (− 565) 5′-CGG ATTTCGTAATAAGGGCA-3′ (− 584). The primer sequences used to amplify the putative ERE of the RAMP3 gene were (13 531 bp from the 5′ end of the first intron) 5′-AGAGTGTACGTGTGGACAGG-3′ (13 550 bp) and (13 735 bp from the 5′ end of the first intron) 5′-CTGTGACAGCAGGAGGACAG-3′ (13 716).

Results

The ADM gene expression pattern correlates well with the uterotrophic effect of estrogen

To select genes that may be involved in the estrogen-induced uterine response, we injected ovariectomized mice with four different doses of estradiol and harvested the uteri 6 h later. The total uterine RNAs were then subjected to DNA microarray analysis, which determined the expression levels of 10 000 genes. The average gene-expression levels were compared with the estrogen dose response of uterine growth (determined by daily injecting ovariectomized mice for a week with the four different estrogen doses and then weighing the whole uteri; Fig. 1A). The genes whose expression levels correlated well with the uterotrophic effect of estrogen were selected.

Of the 10 000 genes examined, 338 had a correlation coefficient of greater than 0·95. The average expression levels of these genes, as determined by the microarray analysis, are shown in Fig. 1B. The 338 selected genes included the ADM gene (Fig. 1C). In addition, the RAMP3 gene displays a similar estrogen-induced expression pattern, as shown by Fig. 1D. This is of interest because RAMP3 modifies the calcitonin gene-related receptor (CRLR), after which CRLR can serve as a receptor for ADM (McLatchie *et al.* 1998). However, CRLR gene expression was unaffected by estrogen treatment (Fig. 1D). The good correlation between ADM and RAMP3 gene expression prompted us to examine the gene-expression regulation of ADM and RAMP3 by estrogen.

ADM and RAMP3 gene expression are activated early after estrogen administration

To examine the temporal changes in ADM gene expression by estrogen exposure, we injected ovariectomized mice with 5 μg/kg b.w. estradiol, harvested the uteri 1, 2, 4 or 6 h later, and subjected the whole uterine RNA to quantitative PCR to evaluate ADM expression. The experiment was performed three times. While ADM gene expression in the uteri varied quite widely 2–4 h after estrogen administration, it was elevated 1 h after estrogen administration and continued to increase thereafter until around 4 h after estrogen administration, after which ADM expression started to decrease (Fig. 2A). Thus ADM gene expression was induced early after estrogen stimulation. RAMP3 gene expression was also induced immediately after estrogen administration and continued until 4 h after estrogen administration (Fig. 2B).

There is also another modifying protein that confers ADM receptor function onto CRLR, namely RAMP2 (McLatchie *et al.* 1998). However, we found RAMP2 expression was not effectively activated by estrogen (Fig. 2C). Moreover, RAMP1, a third CRLR-modifying protein that modifies CRLR to act as a receptor for calcitonin-gene-related peptide (CGRP; McLatchie *et al.* 1998), was substantially repressed by estrogen (Fig. 2C). Furthermore, CGRP, which binds to receptors bearing RAMP3 or RAMP1 (McLatchie *et al.* 1998), was

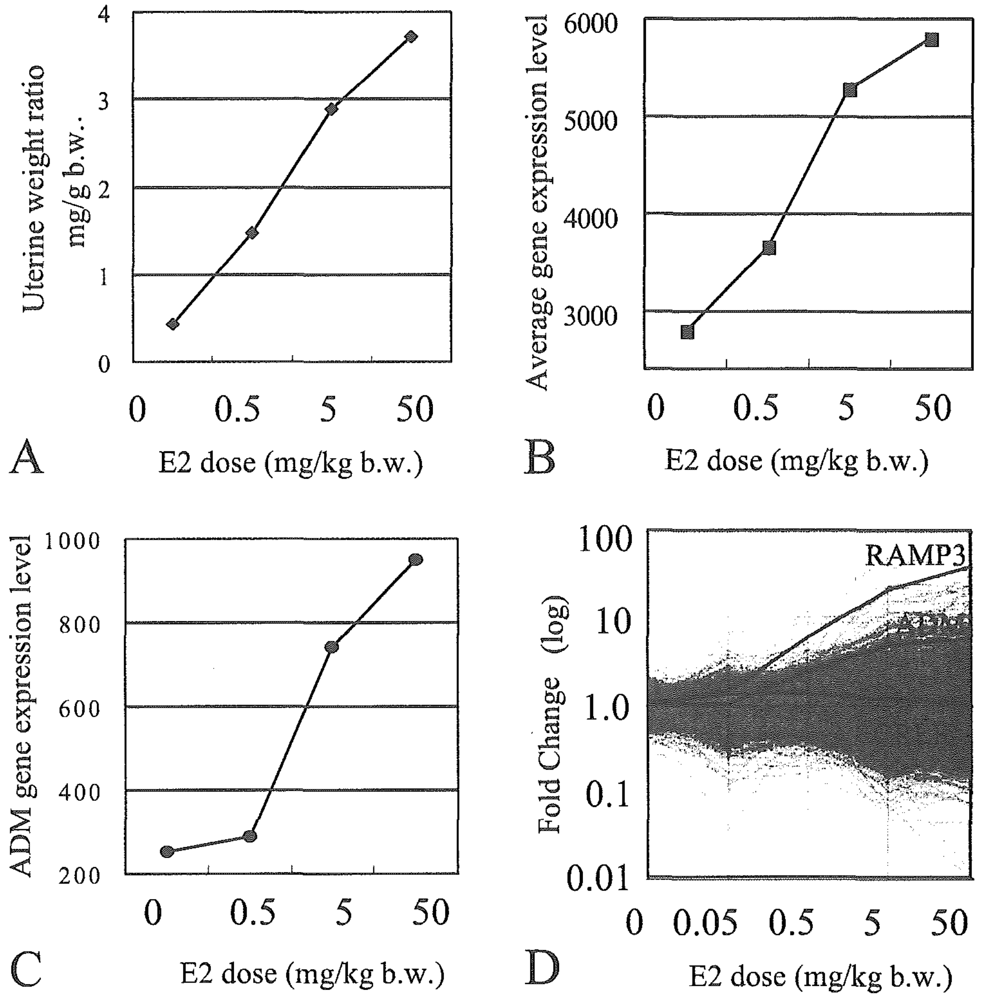

Figure 1 Correlation between estrogen dose-dependent uterine ADM gene expression and the uterotrophic effect of estrogen. (A) Uterotrophic effect of estrogen. Estrogen dose-dependent uterine growth is indicated by a ratio to body weight. (B) Average gene-expression levels of the selected genes; 338 genes whose expression correlated well with the uterotrophic effect were selected and the average gene-expression levels of the 338 genes at the different estrogen doses (calculated from the fluorescent signal intensity of each gene in the DNA microarray analysis) are indicated. (C) Estrogen-dependent gene activation of ADM gene. The gene-expression level of ADM (one of the 338 genes) is indicated. The *x* axis indicates the estradiol dose administered while the *y* axis indicated the gene-expression levels. (D) Estrogen dose-dependent changes in ADM, RAMP3 and CRLR gene expression (solid lines) relative to their expression levels at 0 μg/kg b.w. estradiol (fold expression). Gene expression of all the 10 000 genes examined is shown in gray. While ADM and RAMP3 gene expression were activated by estrogen, CRLR gene expression was not changed. The *x* axis indicates the estradiol dose administered while the *y* axis indicates fold changes in gene expression on a log scale. Note that because of the normalization algorithm, the gray gene expression is not exactly 1·0 but spread around 1·0 at the 0 mg/kg b.w. dose. E2, 17β-estradiol.

expressed in the absence of estrogen at much lower levels than ADM and was unaltered by estrogen administration (data not shown).

To examine how the expression levels of RAMP3 relate to CRLR expression upon estrogen exposure, the ratio of RAMP3 mRNA to CRLR mRNA was calculated from data obtained by quantitative PCR. As indicated by Fig. 1D, CRLR expression is unaltered by estrogen. Although RAMP3 mRNA levels were one-tenth of those of CRLR before estrogen administration, estrogen induced RAMP3 gene transcription, with the result that RAMP3 and CRLR were expressed at equivalent levels 2 h after estrogen administration, shown by mRNA levels. Although protein-level confirmation is

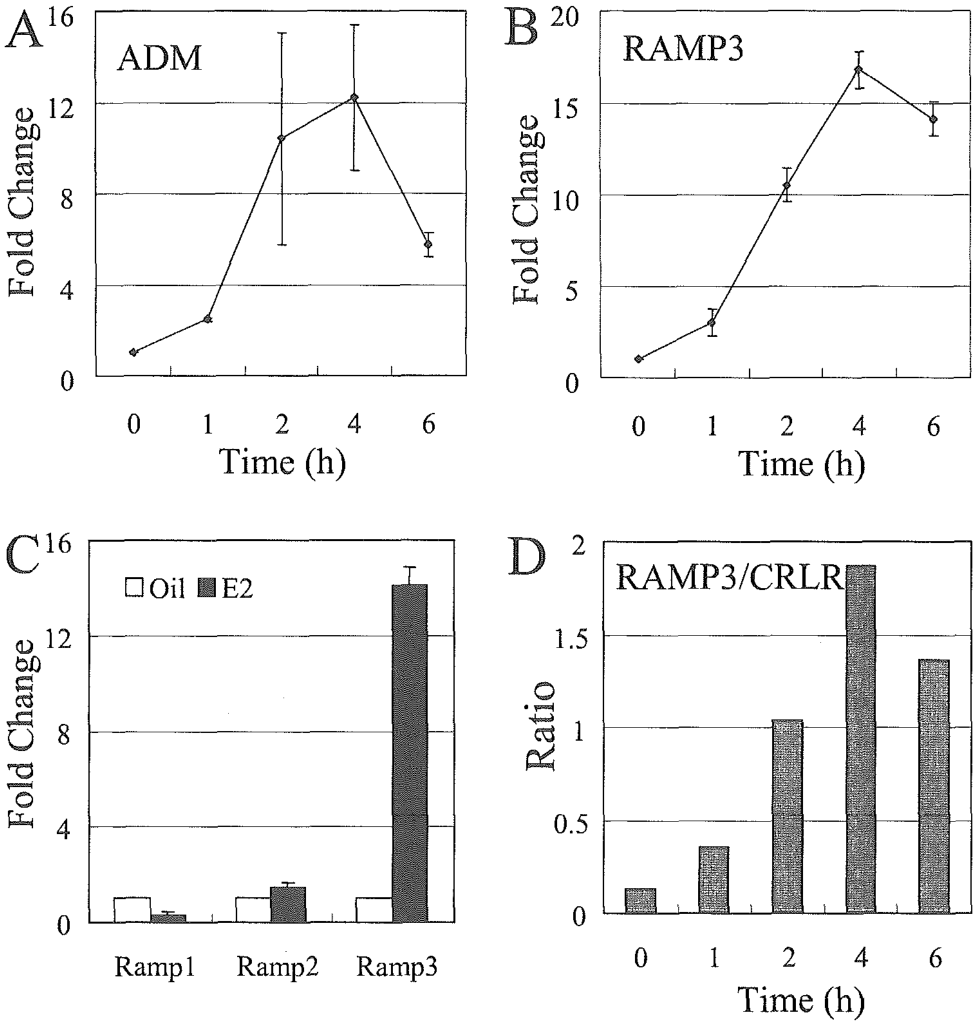

Figure 2 Temporal changes in ADM and RAMP3 gene expression. Ovariectomized mice were injected with 5 μg/kg b.w. estradiol and total uterine RNA was isolated 1, 2, 4 and 6 h later. ADM (A) and RAMP3 (B) gene-expression levels were estimated by quantitative PCR. Shown is the change in expression over time relative to the gene-expression level at 0 h (fold change). Uterine RNA was prepared from three independent experiments and average fold changes and errors are indicated. The *x* axes indicate the time after estrogen administration while the *y* axes indicate fold changes in gene expression. (C) Of the different RAMP genes, only RAMP3 was strongly activated by estradiol. Total uterine RNA was isolated 6 h after injecting 5 μg/kg b.w. estradiol and the change in RAMP gene expression relative to expression in the sesame-oil-injected control uteri was estimated by quantitative PCR. Uterine RNA was prepared from three independent experiments and the average fold changes and errors are indicated. (D) Gene expression of RAMP3 relative to CRLR expression mRNA after estradiol administration was determined by quantitative PCR. The ratio of RAMP3 mRNA to CRLR mRNA was calculated on the basis of the copy numbers of RAMP3 and CRLR mRNA. Approximately equal amounts of mRNA were detected 2 h after estrogen administration. The *x* axis indicates time after estrogen administration while the *y* axis indicates the ratio of RAMP3 mRNA to CRLR mRNA. E2, 17β-estradiol.

essential, the rapid increase of RAMP3 mRNA may affect the properties of the ADM receptor and it appears that the net effect of estrogen exposure is to convert existing CRLR protein into an ADM receptor.

The ER binds to the promoter region of the ADM and RAMP3 genes

Since ADM expression is induced early after estrogen administration, we examined whether the ADM gene

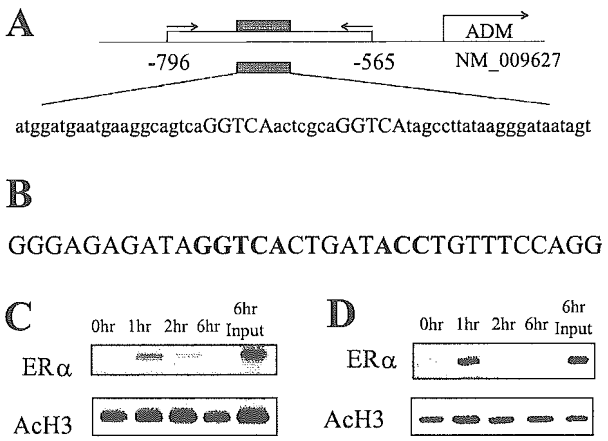

Figure 3 Chromatin immunoprecipitation of the ADM promoter region. (A) Schematic diagram of the ADM promoter region. Q7 The exons of the ADM gene are indicated by boxes. The putative ERE is indicated by a triangle. The nucleotide sequence containing the two direct repeats is indicated (the repeats are indicated by capital letters). (B) Schematic diagram of the RAMP2 gene. Exons of the RAMP3 gene are indicated Q7 by boxes. The putative ERE is indicated by a triangle. The nucleotide sequence containing the putative ERE is indicated (the motif is indicated by capital letters). (C) The ADM promoter region was precipitated by anti-ERα antibody. Chromatin immunoprecipitation was performed on mouse uteri obtained 0, 1, 2 and 6 h after treatment with 5 μg/kg b.w. estradiol. At 1 and 2 h after estrogen administration, the ADM promoter region was precipitated by anti-ERα (upper panel). When chromatin immunoprecipitation using anti-acetylated histone H3 (AcH3) antibody was performed, the ADM promoter region was precipitated both before and after estrogen administration (lower panel). Without specific antibodies, no DNA was amplified (data not shown). (D) The RAMP3 intron was precipitated by anti-ERα antibody. Chromatin immunoprecipitation was performed on mouse uteri obtained 0, 1, 2 and 6 h after treatment with 5 μg/kg b.w. estradiol. At 1 h after estrogen administration, the RAMP3 intron was precipitated by anti-ERα (upper panel). When chromatin immunoprecipitation using anti-acetylated histone H3 antibody was performed, the same region was precipitated both before and after estrogen administration (lower panel). Without specific antibodies, no DNA was amplified (data not shown).

can be directly activated by the ER. Analysis of the sequence upstream of the ADM gene revealed direct repeats of the canonical ERE motif between positions − 744 and − 748 and between positions − 756 and − 760 from the 5′ end of the ADM gene (NM_009627; Fig. 3A). [The actual distance from the transcriptional start site may differ since the transcriptional start site database (http://dbtss/hgc/jp/) showed that the ADM gene has multiple transcriptional start sites located between positions − 9 and − 14.] To examine whether the ER can bind to the element *in vivo*, we performed chromatin-immunoprecipitation assays with uterine DNA obtained 0, 1, 2 and 6 h after treatment with 5 μg/kg b.w. estradiol. A DNA fragment containing the direct repeats was precipitated by the anti-ERα antibody only after estrogen administration (Fig. 3C), suggesting that the ER binds to the promoter region of the ADM gene and activates its transcription. Unlike the ligand-dependent binding of the ER to the ADM promoter, anti-acetylated histone H3 antibody precipitated the direct repeat-bearing DNA fragment even before estrogen was added (0 h). Thus the chromatin structure of the ADM gene is open before the addition of ligand and is accessible to the ER when the ligand is administered. Similarly RMAP3 expression was also activated by estrogen. Although ERE could not be found near the transcription start site, a putative ERE was found in the first intron of RAMP3 gene (13 620 bp from the 5′ end of the first intron; Fig. 3B). Chromatin immunoprecipitation confirmed that the DNA fragment containing the putative ERE could be precipitated by anti-ERα only when estrogen was administered (Fig. 3D). Similar to ADM gene, anti-acetylated histone H3 antibody could precipitate the DNA fragment of RAMP3 even before estrogen administration.

The ADM gene is expressed in uterine stroma cells

To examine the location of ADM gene expression in the uterus, we used *in situ* hybridization to detect ADM mRNA in uteri obtained 6 h after stimulation with 5 μg/kg b.w. estrogen. ADM mRNA was mainly detected in the endometrial stroma (Fig. 4). In contrast to the weak signal in unstimulated ovariectomized uteri, the ADM gene was strongly expressed in the stroma of the estrogen-stimulated uteri. Thus estrogen induces ADM gene expression in the stroma only. Interestingly, the ADM gene was not expressed in the myometrium or epithelial cells. A similar expression pattern was observed for RAMP3, namely weak expression in unstimulated uteri and strong expression in the stroma of estrogen-stimulated uteri. The distribution of RAMP3 mRNA in the stroma was similar to that of CRLR mRNA (data not shown). In contrast, RAMP2 expression did not change after estrogen administration, although its expression was limited to the stroma. Thus the temporal (Fig. 2) and spatial (Fig. 4) expression of the ADM and RAMP3 genes correlated closely.

ADM gene is not activated in ERα-null mice

To examine whether ADM gene activation is dependent on ER, we examined the gene-expression profile of ERα and -β-null mice using DNA microarray. As shown in Fig. 5, ADM was activated in wild-type and ERβ-null mice but not ERα-null mice. This result confirmed that ERα is responsible for the gene activation of ADM. Similarly, RAMP3 was also activated in wild-type and ERβ-null mice but not ERα-null mice. These results suggest that both ADM and RAMP3 genes are regulated by ERα but not ERβ.

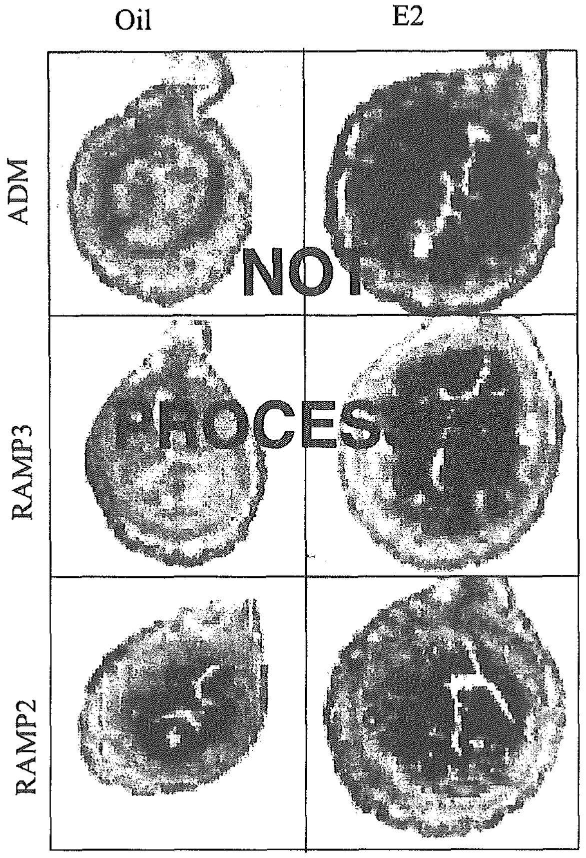

Figure 4 Distribution of ADM, RAMP3 and CRLR mRNAs in estrogen-treated uteri. Ovariectomized mice were given 5 µg/kg b.w. estradiol and their uteri were isolated 6 h later. ADM, RAMP3 and CRLR gene expression was examined by *in situ* hybridization. As a control, mice were injected with sesame oil. The dark-field images were converted to red and merged with bright-field images. E2, 17β-estradiol.

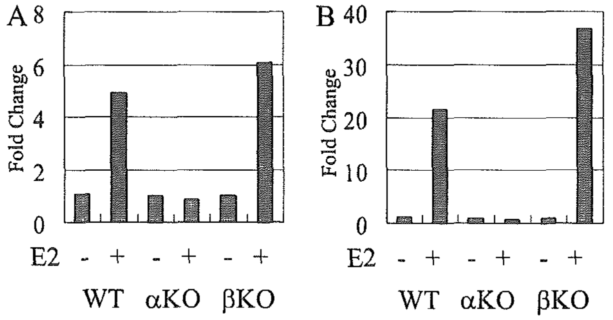

Figure 5 ADM and RAMP3 genes are not activated in ERα-null mice. Female C57BL/6J mice and ERα- and -β-null mice were ovariectomized at 8 weeks of age and injected with either 17β-estradiol (E2, +; 50 µg/kg body weight) or vehicle (−) at 10 weeks of age. The whole uteri were collected 6 h later, and the gene-expression profile was analyzed by DNA microarray. Calculated fold changes are indicated. (A) ADM, (B) RAMP3. WT, wild type; αKO, ERα-knockout; βKO, ERβ-knockout.

Discussion

Since the development of DNA microarray technology, many genes have been listed as being responsive to specific stimuli. However, despite knowing which genes may be activated or repressed by a particular stimulus, the biological significance of this information is still largely lacking. Moreover, many genes identified by DNA microarray analysis have not been validated to respond to the stimulus in question by other methods. In this study, we focused on a gene that is raised from DNA Q5 microarray analysis and showed that the ADM and RAMP3 genes are directly regulated by the ER.

ADM was originally identified as a potent vasorelaxant peptide that is produced by pheochromocytoma cells (Kitamura *et al.* 1993*b*). The human form consists of 52 amino acid residues and the mouse and rat forms consist of 50 amino acid residues (Sakata *et al.* 1993). Subsequent studies revealed that ADM is expressed not only in vascular endothelial cells but also in the adrenal gland (Kitamura *et al.* 1993*a*), kidney (Kitamura *et al.* 1993*a*), heart (Perret *et al.* 1993), bone (Cornish *et al.* 1997) and other tissues. Thus ADM is active in the cardiovascular system (Ishiyama *et al.* 1993), the endocrine system (Yamaguchi *et al.* 1995) and the central nervous system (Wang *et al.* 1995). It is also known to be expressed in the reproductive system as its expression in the uterus has been observed; moreover, uterine ADM expression increases during pregnancy (Di Iorio *et al.* 1999, Michishita *et al.* 1999, Thota *et al.* 2003, Upton *et al.* 1997). Furthermore, studies of the relationship between estrogen and ADM expression in the uterus have revealed a positive correlation between estrogen levels and ADM gene expression (Cameron *et al.* 2002, Ikeda *et al.* 2004, Jerat & Kaufman 1998). Thus ADM appears to participate in phyiological processes affecting the uterus.

Many studies on ADM are mainly concerned with its long-lasting activity in physiological processes such in hypotension (Kitamura *et al.* 1995), bone development (Cornish *et al.* 2003) and gestation (Thota *et al.* 2003, Upton *et al.* 1997). With regard to the reproductive system, ADM has been reported to generally act as a relaxant or vasodilator (Makino *et al.* 1999, Yanagita *et al.* 2000). However, the data presented in this paper show that locally expressed ADM can also function in the uterus in a more transient manner, since estrogen-induced ADM gene expression in the uterus only occurs in the stroma over a short period. Interestingly, although ADM, RAMP3 and CRLR are only expressed in the stromal cells, this kind of local effect of ADM has been observed in many tissues (Kato *et al.* 1997, Martinez *et al.* 1997, Nishimura *et al.* 1997, Seguchi *et al.* 1995, Takahashi *et al.* 1997), including the uterus (Nikitenko *et al.* 2000). Further analysis of ADM signaling is needed to clarify the effects of estrogen on uterine growth.

In the present study, we also demonstrated by chromatin-immunoprecipitation assays that the ADM promoter is recognized by the ER in a ligand (estrogen)-dependent manner. Although we used whole uteri for this analysis, these results appear to reflect the effects of ADM in the stroma, which is abundant in the uterus. Our data show that rapid binding of the ER directly to the ADM promoter may be important for ADM gene activation. A putative ERE was also detected in the RAMP3 gene, though it was found in the first intron of the gene. Chromatin immunoprecipitation confirmed that this region could be recognized by ERα in a ligand-dependent manner.

In summary we have shown that ADM and RAMP3 are genes that are directly activated by the ER, and that ADM and RAMP3 are directly recognized by the ERα. Similarities between the gene-activation patterns of ADM and RAMP3 suggest that a combination of RAMP3 and CRLR functions as an ADM receptor after estrogen administration. Although functional analysis of these genes is essential, the early activation of ADM after estrogen administration suggests that ADM plays important roles in the reproductive system in different ways: first, in processes such as pregnancy, where its involvement and expression are prolonged (Di Iorio *et al.* 1999, Michishita *et al.* 1999, Thota *et al.* 2003, Upton *et al.* 1997), and second, in processes such as estrogen-induced uterine growth, where its involvement and expression are transient.

Acknowledgements

This work was supported in part by a Core Research for Evolutional Science research grant from the Japan Science and Technology Cooperation, a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, a Health Sciences Research Grant from the Ministry of Health, Labour and Welfare of Japan, a research grant from the Ministry of Environment, Japan, and a research grant from New Energy and Industrial Technology Development Organization (NEDO).

Q6

References

Cameron VA, Autelitano DJ, Evans JJ, Ellmers LJ, Espiner EA, Nicholls MG & Richards AM 2002 Adrenomedullin expression in rat uterus is correlated with plasma estradiol. *American Journal of Physiology Endocrinology & Metabolism* **282** E139–E146.

Cornish J, Callon KE, Coy DH, Jiang NY, Xiao L, Cooper GJ & Reid IR 1997 Adrenomedullin is a potent stimulator of osteoblastic activity in vitro and in vivo. *American Journal of Physiology Endocrinology & Metabolism* **273** E1113–E1120.

Cornish J, Naot D & Reid IR 2003 Adrenomedullin–a regulator of bone formation. *Regulatory Peptides* **112** 79–86.

Di Iorio R, Marinoni E, Letizia C, Villaccio B, Alberini A & Cosmi EV 1999 Adrenomedullin production is increased in normal human pregnancy. *European Journal of Endocrinology* **140** 201–206.

Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P & Mark M 2000 Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development* **127** 4277–4291.

Ikeda K, Arao Y, Otsuka H, Kikuchi A & Kayama F 2004 Estrogen and phytoestrogen regulate the mRNA expression of adrenomedullin and adrenomedullin receptor components in the rat uterus. *Molecular & Cell Endocrinology* **223** 27–34.

Ishiyama Y, Kitamura K, Ichiki Y, Nakamura S, Kida O, Kangawa K & Eto T 1993 Hemodynamic effects of a novel hypotensive peptide, human adrenomedullin, in rats. *European Journal of Pharmacology* **241** 271–273.

Jerat S & Kaufman S 1998 Effect of pregnancy and steroid hormones on plasma adrenomedullin levels in the rat. *Canadian Journal of Physiology & Pharmacology* **76** 463–466.

Kato H, Shichiri M, Marumo F & Hirata Y 1997 Adrenomedullin as an autocrine/paracrine apoptosis survival factor for rat endothelial cells. *Endocrinology* **138** 2615–2620.

Kitamura K, Kangawa K, Matsuo H & Eto T 1995 Adrenomedullin. Implications for hypertension research. *Drugs* **49** 485–495.

Kitamura K, Sakata J, Kangawa K, Kojima M, Matsuo H & Eto T 1993*a* Cloning and characterization of cDNA encoding a precursor for human adrenomedullin. *Biochemical & Biophysics Research Communications* **194** 720–725.

Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H & Eto T 1993*b* Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochemistry & Biophysics Research Communications* **192** 553–560.

Makino I, Shibata K, Makino Y, Kangawa K & Kawarabayashi T 1999 Adrenomedullin attenuates the hypertension in hypertensive pregnant rats induced by N(G)-nitro-L-arginine methyl ester. *European Journal of Pharmacology* **371** 159–167.

Martinez A, Miller MJ, Catt KJ & Cuttitta F 1997 Adrenomedullin receptor expression in human lung and in pulmonary tumors. *Journal of Histochemistry & Cytochemistry* **45** 159–164.

McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG & Foord SM 1998 RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* **393** 333–339.

Michishita M, Minegishi T, Abe K, Kangawa K, Kojima M, Ibuki Y, Upton PD, Austin C, Taylor GM, Nandha KA *et al.* 1999 Expression of adrenomedullin (ADM) and its binding sites in the rat uterus: increased number of binding sites and ADM messenger ribonucleic acid in 20-day pregnant rats compared with nonpregnant rats. *Obstetics & Gynecology* **93** 66–70.

Nikitenko LL, MacKenzie IZ, Rees MC & Bicknell R 2000 Adrenomedullin is an autocrine regulator of endothelial growth in human endometrium. *Molecular & Human Reproduction* **6** 811–819.

Nishimura J, Seguchi H, Sakihara C, Kureishi Y, Yoshimura H, Kobayashi S & Kanaide H 1997 The relaxant effect of adrenomedullin on particular smooth muscles despite a general expression of its mRNA in smooth muscle, endothelial and epithelial cells. *British Journal of Pharmacology* **120** 193–200.

Perret M, Broussard H, LeGros T, Burns A, Chang JK, Summer W, Hyman A & Lippton H 1993 The effect of adrenomedullin on the isolated heart. *Life Science* **53** PL377–PL379.

Sakata J, Shimokubo T, Kitamura K, Nakamura S, Kangawa K, Matsuo H & Eto T 1993 Molecular cloning and biological activities of rat adrenomedullin, a hypotensive peptide. *Biochemistry & Biophysics Research Communications* **195** 921–927.

Seguchi H, Nishimura J, Kobayashi S, Kumazawa J & Kanaide H 1995 Autocrine regulation of the renal arterial tone by adrenomedullin. *Biochemistry & Biophysics Research Communications* **215** 619–625.

Szego CM & Davis JS 1967 Adenosine 3′,5′-monophosphate in rat uterus: acute elevation by estrogen. *PNAS* **58** 1711–1718.

Takahashi K, Satoh F, Sone M, Murakami O, Sasano H, Mouri T & Shibahara S 1997 Expression of adrenomedullin mRNA in the human brain and pituitary. *Peptides* **18** 1051–1053.

Thota C, Gangula PR, Dong YL & Yallampalli C 2003 Changes in the expression of calcitonin receptor-like receptor, receptor activity-modifying protein (RAMP) 1, RAMP2, and RAMP3 in rat uterus during pregnancy, labor, and by steroid hormone treatments. *Biology of Reproduction* **69** 1432–1437.

Upton PD, Austin C, Taylor GM, Nandha KA, Clark AJ, Ghatei MA, Bloom SR & Smith DM 1997 Expression of adrenomedullin (ADM) and its binding sites in the rat uterus: increased number of binding sites and ADM messenger ribonucleic acid in 20-day pregnant rats compared with nonpregnant rats. *Endocrinology* **138** 2508–2514.

Wang X, Yue TL, Barone FC, White RF, Clark RK, Willette RN, Sulpizio AC, Aiyar NV, Ruffolo RR Jr & Feuerstein GZ 1995 Discovery of adrenomedullin in rat ischemic cortex and evidence for its role in exacerbating focal brain ischemic damage. *PNAS* **92** 11480–11484.

Watanabe H, Suzuki A, Mizutani T, Khono S, Lubahn DB, Handa H & Iguchi T 2002 Genome-wide analysis of changes in early gene expression induced by oestrogen. *Genes Cells* **7** 497–507.

Watanabe H, Suzuki A, Kobayashi M, Lubahn DB, Handa H & Iguchi T 2003 Similarities and differences in uterine gene expression patterns caused by treatment with physiological and non-physiological estrogens. *Journal of Molecular Endocrinology* **31** 487–497.

Yamaguchi T, Baba K, Doi Y & Yano K 1995 Effect of adrenomedullin on aldosterone secretion by dispersed rat adrenal zona glomerulosa cells. *Life Science* **56** 379–387.

Yanagita T, Yamamoto R, Sugano T, Kobayashi H, Uezono Y, Yokoo H, Shiraishi S, Minami SI & Wada A 2000 Adrenomedullin inhibits spontaneous and bradykinin-induced but not oxytocin- or prostaglandin F(2 alpha)-induced periodic contraction of rat uterus. *British Journal of Pharmacology* **130** 1727–1730.

Zor U, Koch Y, Lamprecht SA, Ausher J & Lindner HR 1973 Mechanism of oestradiol action on the rat uterus: independence of cyclic AMP, prostaglandin E2 and beta-adrenergic mediation. *Journal of Endocrinology* **58** 525–533.

Received 26 July 2005
Accepted 17 October 2005

'Not Processed' Figures

Figures marked as 'Not Processed' are of low quality/resolution (*more specific details may be outlined in the list below*), please resupply

Specific Queries (see Q in text)

1. what does this - symbol mean here?
2. please indicate the orientation of each primer
3. define OCT in full
4. town?
5. your meaning here is unclear: (i) which gene do you mean? ADM? Please mention it by name. (ii) what do you mean by 'raised from DNA microarray analysis'? Do you mean that the gene is shown by DNA microarray analysis to be increased in expression as a result of a stimulus? Please clarify
6. Is there any conflict of interest in this research? If not, it is journal policy to insert the following declaration in the Funding/Acknowledgements section: The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work. Is this OK?
7. what's the difference between white and black boxes?

The Journal of Toxicological Sciences,
Vol.30, No.2, 75-89, 2005

SEX STEROID HORMONE RECEPTORS IN THE DEVELOPING FEMALE REPRODUCTIVE TRACT OF LABORATORY RODENTS

Akinobu OKADA[1*], Tomomi SATO[2], Yasuhiko OHTA[3,4] and Taisen IGUCHI[4,5]

[1]*Safety Research Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd.,*
1-1-8 Azusawa, Itabashi-ku, Tokyo 174-8511, Japan
[2]*Graduate School of Integrated Science, Yokohama City University,*
22-2 Seto, Kanazawa-ku, Yokohama 236-0027, Japan
[3]*Department of Veterinary Science, Faculty of Agriculture, Tottori University,*
4-104 Koyama-Minami, Tottori 680-8553, Japan
[4]*CREST, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi 332-0012, Japan*
[5]*Okazaki Institute for Integrative Bioscience, National Institute for Basic Biology, National Institutes of Natural Science,*
and Department of Molecular Biomechanics, School of Life Science, The Graduate University for Advanced Studies,
5-1 Higashiyama, Myodaiji, Okazaki 444-8787, Japan

(Received January 7, 2005; Accepted February 8, 2005)

ABSTRACT — Many chemicals released into the environment potentially disrupt the endocrine system in wildlife and humans. Some of these chemicals exhibit estrogenic activity by binding to the estrogen receptors. The developing organism is particularly sensitive to estrogenic chemicals during the critical period in which the induction of long-term changes and persistent molecular alterations in female reproductive tracts occur. Perinatal mouse and rat models can be utilized as indicators for determining the consequences of exposure to exogenous estrogenic agents, including possible xenoestrogens or environmental endocrine disruptors. Estrogen receptors (ER) and estrogen responsive genes, therefore, need to be identified in order to understand the molecular basis of estrogenic actions. Recent identifications of ER subtypes and isoforms make understanding target organ responses to these estrogenic chemicals even more difficult. Indeed, many reports suggest that these chemicals do affect the reproductive and developmental processes of female laboratory rodents that had been perinatally exposed, and that interactions between sex steroid hormone receptors occur. Much information concerning the expression of sex steroid receptors in rodents has been reported concerning the normal development of the Müllerian duct. Thus, accumulated information on the expression of ER subtypes and isoforms as well as that of progesterone and androgen receptors in laboratory rodents is herein reviewed, in addition to the presentation of our own data.

KEY WORDS: Estrogen receptor, Progesterone receptor, Androgen receptor, Reproductive tracts, Müllerian duct, Endocrine disruption

INTRODUCTION

Recently, there has been considerable concern about the potential endocrine-disrupting effects of chemicals released into the environment in wildlife and humans (Colborn and Clement, 1992). Chemicals that have estrogenic activity are termed xenoestrogens or endocrine disruptors. They are thought to mimic or disturb the action of estrogen and many of them are known to possess estrogen receptor-binding activity (Danzo, 1997). This fact brings to mind historical work by Herbst *et al.* done in the early seventies (1971) when a close correlation between the occurrence of vaginal clear cell adenocarcinoma in young women and early intrauterine exposure to diethylstilbestrol (DES), a synthetic estrogen, was demonstrated (Herbst and Bern,

---

Correspondence: Akinobu OKADA
* Present address: Drug Safety Research Laboratories, Astellas Pharma Inc., 1-6, Kashima 2-chome, Yodogawa-ku, Osaka 532-8514, Japan.

1981). Similarly, during prenatal and neonatal development of the paramesonephric or Müllerian duct, estrogen exposure has been found to induce a variety of abnormalities in the Müllerian derivatives such as uncoiling of the oviduct and uterine hyperplasia in addition to neoplasia, vaginal hyperplasia and ovary-independent cornification with an abnormal cell proliferation rate in mice and rats (Dunn and Green, 1963; Takasugi and Bern, 1964; Iguchi and Takasugi, 1987; Newbold *et al.*, 1983; Iguchi, 1992; Sato *et al.*, 1994, 2004; Okada *et al.*, 2001). Laboratory rodents exposed perinatally to estrogens, therefore, provide a model for the exploration of the consequences of xenoestrogen exposure in humans, since rodent genital tract development at birth is similar to that of the human fetus at the end of the first trimester.

Recent developments in molecular and cellular biology techniques have identified many subtypes and isoforms of sex steroid hormone receptors and the complex interaction between them. The specific importance of subtypes and isoforms of estrogen receptor (ER) and progesterone receptor (PR) in reproductive and non-reproductive tissues was proposed in ER or PR knockout mice (Lubahn *et al.*, 1993; Krege *et al.*, 1998; Couse and Korach, 1999; Couse *et al.*, 1999; Conneely and Lydon, 2000; Dupont *et al.*, 2000; Conneely *et al.*, 2003). However, reproductive tract development is normal during the prenatal and neonatal stages in the knockout mice, suggesting that ER and PR signals are not necessary for the morphogenesis of the female reproductive tract. Nevertheless, as described above, perinatal administration of estrogens exerts teratogenic and carcinogenic effects on female rodent reproductive tracts. Findings in estrogen receptor α knockout (αERKO) mice and in transgenic mice which overexpress ERα lead to the speculation that the teratogenic and carcinogenic effects of estrogens are mediated through ERα (Couse *et al.*, 1997, 2001). There have been accumulating reports regarding the expression pattern of sex steroid hormone receptors in the developing female reproductive tracts in mice and rats. This information allows us to understand which cells and tissues are targets for physiological and exogenous estrogens. The prenatal and neonatal ontogenies of sex steroid hormone receptors in female reproductive tracts have been reported (Pasqualini and Sumida, 1986; Greco *et al.*, 1993). However, experiments have been performed using radio-labeled ligands or immunohistochemistry with the antibody, both of which are non-specific to the receptor subtypes or isoforms (Murakami *et al.*, 1990). Thus, in order to have a better understanding of the molecular and cellular mechanisms underlying effects of sex steroid hormones during the development of female reproductive tracts, this article will review recent progress in the subtype and isoform expression of ER in laboratory rodents, as well as those of PR and androgen receptor (AR).

PR is one of the well-studied estrogen-regulated genes that contain estrogen response elements (ERE) (Kastner *et al.*, 1990; Kraus *et al.*, 1994). Previous reports demonstrated that progesterone-binding sites and PR expression were regulated by the estrogens in female reproductive tracts (Ennis and Stumpf, 1988; Ohta *et al.*, 1996; Okada *et al.*, 2002b). Kurita and coworkers (2000) showed mechanistic evidence of PR regulation by estradiol (E2) in mouse uterine and vaginal epithelia *in vivo* in tissue recombinants made with epithelium and stroma from wild-type and/or αERKO mice. They suggested paracrine regulation via stromal ERα and direct regulation via epithelial ERα in mouse uterus and vagina, respectively. Androgens also have uterotrophic effects in intact and ovariectomized immature female rats (Armstrong *et al.*, 1976), and testicular feminized male (T*fm*/T*fm*) mice show impaired reproductive performance (Lyon and Glenister, 1980), both of which suggest the importance of androgens in female reproduction (Kowalski *et al.*, 2004). Weihua *et al.* (2002) have recently reported the essential role of AR in estrogen-induced uterine epithelial cell proliferation in rats, and they indicated that stromal AR amplified the ERα signal by induction of insulin-like growth factor I (IGF-I), which is known to be produced in stromal cells and induce epithelial cell proliferation in a paracrine fashion. Moreover, direct inhibitory effects of the ERα/AR heterodimer on both ERα and AR transactivational properties have been reported (Panet-Raymond *et al.*, 2000). Thus, identification of regional and cellular AR and PR localization may allow a better understanding not only of the role of AR and PR, but also the mechanism of estrogen action in female reproductive tracts.

A brief description of the development of the female reproductive tract

In mammals, both the paramesonephric or Müllerian duct and the mesonephric or Wolffian duct are situated within the genital ridge. There are two ducts present during fetal development. The Müllerian duct develops in the cranial to caudal direction along with the Wolffian duct during gonad formation (Dohr and Tarmann, 1984; Byskov and Høyer, 1994). After gonad differentiation, androgens produced by Leydig cells induce Wolffian duct development, and Müllerian inhibiting substance (MIS) produced by Sertoli cells induces Müllerian duct degeneration in male fetuses. In female fetuses, Müllerian duct development and Wolffian duct degeneration occur due to a lack of androgens and MIS after gonadal differentiation (Josso and Picard, 1986). Thus, male reproductive organs such as the epididymis, vas deferens, and seminal vesicles arise from the Wolffian duct, and female reproductive organs such as the oviduct, uterus, and upper vagina arise from the Müllerian duct.

The Müllerian duct is comprised of epithelial cells surrounded by mesenchymal cells. The pattern of cell proliferation of the Müllerian duct has been demonstrated in prenatal, immature, and adult rodents (McCormack and Glasser, 1980; Quarmby and Korach, 1984; Li, 1994; Okada *et al.*, 2001). In tissue recombination experiments, epithelial-mesenchymal interactions are critically important for the proper differentiation of the Müllerian duct derivatives (Kurita *et al.*, 2001). At birth, Müllerian epithelium is negative for uterine and vaginal epithelial markers, and uterine and vaginal gene expression patterns are induced in neonatal Müllerian epithelium by the respective mesenchymes. Kurita *et al.* (2001) also showed that functional differentiation of uterine and vaginal epithelia required organ-specific mesenchymal/stromal factors, but mesenchymal/stromal signals regulating proliferation of epithelial cells in the uterus and vagina appeared to be nonspecific. Since it is well known that the functional differentiation of uterine and vaginal epithelia is regulated by E2 and progesterone (P4), mesenchymal/stromal signals for regulating functional epithelial differentiation may be controlled through mesenchymal/stromal ERs and PRs. Thus, temporal expressions of these receptors may be helpful for understanding Müllerian duct development.

Subtypes and isoforms of estrogen and progesterone receptors

Estrogen, progesterone, and androgen actions are mediated by ERs, PRs, and AR, respectively, which belong to the nuclear receptor superfamily of ligand-inducible transcription factors (Mangelsdorf *et al.*, 1995). The steroid hormone receptor-ligand complex binds target DNA at hormone response elements (HREs) to transcribe various downstream genes such as oncogenes and genes encoding growth factors. In rats, two subtypes of ERs, classical ERα (Koike *et al.*, 1987) and novel ERβ (Kuiper *et al.*, 1996) have been identified. ERβ was also cloned in mice (Tremblay *et al.*, 1997) and humans (Mosselman *et al.*, 1996), showing high homology between the species. E2 bound ERα with a higher affinity than ERβ and increased ERα transcriptional activity at the EREs in a reporter assay (Kuiper *et al.*, 1997; Pettersson *et al.*, 2000). In addition to these differences in transcriptional activity, ERα and ERβ can have entirely opposite transcriptional effects at activating protein-1 (AP-1) sites, depending on the ligands (Paech *et al.*, 1997). Moreover, the role of ERβ as a negative regulator of ERα has been suggested in mouse uterus (Weihua *et al.*, 2000). Five isoforms of ERβ mRNA, ERβ1 (ERβ), ERβ2, ERβ1-δ3, ERβ2-δ3, and ERβ1-δ4, have been reported in various rat tissues as products of alternative splicing (Maruyama *et al.*, 1998; Petersen *et al.*, 1998; Price *et al.*, 2000). Only ERβ1 and ERβ2, which has an additional 18 amino acids in the ligand binding domain of ERβ1, have the ability to bind both ligand and ERE. However, ERβ2 has an apparently lower binding affinity for E2 as compared to ERβ1 (Petersen *et al.*, 1998). Therefore, ERβ2 can act as a dominant negative regulatory partner during heterodimerization with ERα or ERβ1. This action has been demonstrated during rat mammary gland development (Saji *et al.*, 2001).

Two PR isoforms, PR-A and PR-B, are produced from a single gene by transcription at two distinct promoter sites (Horwitz and Alexander, 1983; Kastner *et al.*, 1990; Savouret *et al.*, 1990). The expression of two protein isoforms from the one gene is conserved in several species, including mice, rats, and humans (Lessey *et al.*, 1983; Schott *et al.*, 1991; Kraus *et al.*, 1993). The human PR-A isoform differs from the PR-B in that it lacks 164 amino acids on its N terminus. The ratio of isoforms varies depending on the reproductive tissues during development (Shyamala *et al.*, 1990) and the estrous cycle (Mangal *et al.*, 1997). PR-A- and PR-B-deficient mice suggest the different actions of each PR isoform *in vivo* (Lubahn *et al.*, 1993; Krege *et al.*, 1998; Couse and Korach, 1999; Couse *et al.*, 1999; Conneely and Lydon, 2000; Dupont *et al.*, 2000; Conneely *et al.*, 2003).

ONTOGENIC EXPRESSION OF ESTROGEN RECEPTORS

During prenatal development of the Müllerian duct

Immunohistochemistry of ERα and ERβ showed differential expression of the two ER subtypes in the ovaries and uterus of adult rats (Shughrue *et al.*, 1998; Hiroi *et al.*, 1999; Sar and Welsch, 1999; Wang *et al.*, 1999; Mowa and Iwanaga, 2000a; Pelletier *et al.*, 2000). In female rodents, expression of ERα was detected in both the epithelium and stroma of the uterus and vagina (Sato *et al.*, 1992, 1996; Ohta *et al.*, 1993, 1996; Graham and Clarke, 1997; Mowa and Iwanaga, 2000a; Shughrue *et al.*, 1998; Wang *et al.*, 1999). ERβ is reportedly expressed at a lower level than ERα in the uterus and vagina of adult mice and rats (Mowa and Iwanaga, 2000a; Shughrue *et al.*, 1998; Wang *et al.*, 1999). ER is expressed in the Müllerian duct of fetal mice (Greco *et al.*, 1991; Wu *et al.*, 1992; Greco *et al.*, 1993; Lemmen *et al.*, 1999; Jefferson *et al.*, 2000; Nielsen *et al.*, 2000) and rats (Mowa and Iwanaga, 2000b). Greco and co-workers (1991) reported that ER immunoreactivity was observed in epithelial cells of female mouse reproductive tract at 15 days post-coitus (dpc), but was occasionally observed at 17 dpc and on the day of birth. The results of immunohistochemistry with a monoclonal antibody specific to ERα in mice (Nielsen *et al.*, 2000), and *in situ* hybridization with a radio-labeled probe specific to ERα in rats (Mowa and Iwanaga, 2000b) demonstrate that ERα localization varies with the region of the Müllerian duct involved. Nielsen *et al.* (2000) detected a faint immunopositive signal in the nuclei of the surrounding cells of the Müllerian duct as early as 11.5 dpc in mice. Epithelial ERα was first observed in the proximal oviduct region at 13.5 dpc. At the mRNA level, expression of ERα was exhibited in the oviductal epithelium and all mesenchyme at 17 dpc. It was expressed in the uterine and vaginal epithelia on postnatal days. The semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) study showed that the ERα mRNA level in the Müllerian duct at 21.5 dpc was 4.4-fold higher than that at 15.5 dpc in rats (Okada *et al.*, 2002a). In immunohistochemical evaluations, ERα protein showed a similar expression pattern to its mRNA (Table 1). In the oviduct region at 15.5 dpc, mesenchymal ERα staining could be detected, but epithelial staining was only slight. Epithelial and mesenchymal ERα levels within the oviduct region increased gradually from 15.5 dpc to 19.5 dpc. Uterine and vaginal ERα levels continually increased within the mesenchyme throughout gestational development and reached marked intensity levels by 21.5 dpc. However, epithelial ERα staining was slight or absent from the uterine and vaginal regions throughout gestation until 21.5 dpc. Although this study evaluated ERα expression in the upper vagina, which originated from the Müllerian duct, earlier ERα expression at 16 dpc was detected in the epithelium of the lower vagina, which was derived from the urogenital sinus in mice (Kurita *et al.*, 2001). These observations clearly demonstrate that ERα is expressed region- and cell type-dependently during the prenatal development of the Müllerian duct. The patterns of ERα localization suggest potential tissue-specific mechanisms by which estrogenic chemicals may influence Müllerian duct cell growth and differentiation.

No expression of ERβ1 (ERβ) was detected in the mouse uterus at 16 dpc in the ribonuclease protection assay (RPA) (Jefferson *et al.*, 2000). Two *in situ* hybridization studies revealed similar negative results in the epithelium and mesenchyme of the Müllerian duct throughout the prenatal period in rats and mice (Lemmen *et al.*, 1999; Mowa and Iwanaga, 2000b). However, in the RT-PCR study, rat Müllerian duct ERβ1 and ERβ2 mRNAs were detected and found to be expressed at constant levels from 15.5 dpc to 21.5 dpc (Okada *et al.*, 2002a). Expression levels of ERβ1 and ERβ2 mRNA were much lower than that of ERα. Comparison of ERβ1 and ERβ2 expression levels indicated that ERβ2 mRNA expression is significantly greater than that of ERβ1 in the 19.5 dpc Müllerian duct. The tissue-specific expression ratio of ERβ1 ver-

Table 1. Ontogenetic immunolocalization of ERα in the fetal female rat reproductive tract[a].

Tissue	Cell type	Prenatal days			
		15.5	17.5	19.5	21.5
Oviduct	Epithelium	±	+	++	++
	Mesenchyme	–	±	+	+
Uterus	Epithelium	–	–	–	–
	Mesenchyme	–	±	+	++
Upper vagina	Epithelium	NF	–	–	±
	Mesenchyme	NF	±	+	++

[a]: Data from Okada *et al.* (2002a)

++: marked, +: moderate, ±: slight, –: negative, NF: Not formed.

sus ERβ2 has been described in various rat tissues; approximately 1:1 in prostate, ovary, muscle, and pituitary, and 2- to 6-fold expression of ERβ1 compared to that of ERβ2 in tissues of the nervous system (Petersen *et al.*, 1998). Recently, the importance of ERβ2 as a negative regulator of ERα has been demonstrated in the rat mammary gland in which ERβ2 levels are comparable or higher than those for ERβ1 (Saji *et al.*, 2001). Although both ERβ1 and ERβ2 levels are obviously higher than ERα in the mammary gland, both isoforms were extremely low in rat Müllerian duct compared to ERα, implying insufficient amounts of ERβs acting as a negative regulator of ERα in the rat Müllerian duct. The physiological significance of ERβ2 in rat Müllerian duct is unknown, but different ratios of ERα/ERβ1/ERβ2 among tissues may suggest tissue-specific regulation depending on the ER subtypes and isoforms. Taken together, these results indicate that ERα is likely a dominant ER subtype during Müllerian duct development. Furthermore, region-specific ERα expression firmly indicates that functional differentiation within the Müllerian duct occurs before morphological differentiation during the neonatal period, and that regional targets for chemicals that may act via or influence ER-mediated mechanisms are specific during late gestational Müllerian duct differentiation.

During neonatal development of the oviduct

The mammalian oviduct, or fallopian tube, is part of the female reproductive tract that has a fundamental role in gamete transport, fertilization, and subsequent early embryo development (Jansen, 1984). During neonatal development, the oviduct forms a coiled structure and is composed of four different regions: the infundibulum (INF), ampulla (AMP), isthmus (IST) and uterotubal junction (UTJ). Depending on the oviductal region, there are at least two types of epithelial cells: ciliated epithelial cells and nonciliated or secretory epithelial cells. Growth and development of these cell types are under regulation by the sex steroid hormones E2 and P4 (Abe and Oikawa, 1993). ERα and ERβ expression in the rat oviduct has been reported (Saunders *et al.*, 1997; Sar and Welsch, 1999; Mowa and Iwanaga, 2000a, 2000b; Wang *et al.*, 2000), but the predominant ERα expression was shown in the neonatal oviduct. Oviductal ERβ expression was low in rats (Saunders *et al.* 1997, Mowa and Iwanaga, 2000a, 2000b) and mice (Couse *et al.*, 1997), while Sar and Welsch (1999) and Jefferson *et al.* (2000) failed to detect ERβ immunopositive cells in the oviducts of rats or mice, respectively, at all. In a quantitative real-time RT-PCR study, oviductal ERα mRNA increased until neonatal day (ND) 3, and was maintained at a high constant level through ND 20. However, ERβ mRNA was detected in the oviduct in a low and constant manner, as compared with ERα, throughout pre- and postnatal development (Okada *et al.*, 2003). In the immunohistochemical study of cell- and region-specificity (Table 2), epithelial ERα was weak at birth (ND 0) and exhibited moderate stainings from ND 3 to 20. However, some ERα negative cells were present in the epithelium of the INF/AMP region after ND 10. Stromal cells showed ERα staining at weak, moderate, and marked levels at ND 0, from ND 3 to 10, and ND 15 and 20, respectively, and no marked difference in ERα staining was noted between regions in the oviduct. Moderate ERα staining was also found in muscle cells of the IST/UTJ region from ND 7 to 20. ERβ immunoreactivity was indistinguishable in the neonatal oviduct. Thus, abundant ERα may be a major ER subtype and play an essential role in the development and function of the neonatal rat oviduct.

As described above, in the developing rat oviduct, ERα was expressed in both epithelial and stromal cells, and the staining intensity and mRNA level increased with the growth of the neonates. With the increases in these receptors during the early postnatal period, immunoreactivity for β-tubulin IV, a cilia marker protein, in the rat oviduct appeared between ND 5 and ND 7. Komatsu and Fujita (1978) have reported in their electron-microscopy study that the differentiation of ciliated cells, which is believed to be elicited by the initiation of endogenous estrogen production, occurs in the mouse oviduct on ND 5. Furthermore, neonatal E2 administration accelerated cilia formation in the mouse and rat oviduct (Eroschenko, 1982; Okada *et al.*, 2004a, 2004b). Although the stimulation of ER signaling accelerates the differentiation process of ciliated epithelial cells, the presence of cilia in the αERKO mouse oviduct suggests that it is not fundamentally required for this event in the ERα-negative ciliated epithelial cells of the adult oviduct (Okada *et al.*, 2004a). Double immunohistochemical staining for ERα with β-tubulin IV was reported in the determination of surface epithelial cell types expressing ERα (Okada *et al.*, 2003). The ciliated cells were positive for β-tubulin IV, while the nonciliated (secretory) cells were negative for it. ERα was selectively observed in nonciliated epithelial cells, but not in ciliated epithelial cells of the INF/AMP. In contrast, almost all epithelial cells in the UTJ/IST were negative

Table 2. Ontogenetic immunolocalization of ERα, ERβ, PR-A+B, and AR in the prenatal and neonatal female rat oviduct[a].

	Cell type	Prenatal days		Neonatal days										
		15	19	0	3	5	7		10		15		20	
							INF/AMP	IST/UTJ	INF/AMP	IST/UTJ	INF/AMP	IST/UTJ	INF/AMP	IST/UTJ
ERα	Epi	±	+	+	++	++	++	++	++[b]	++	++[b]	++	++[b]	++
	Str	–	±	+	++	++	++	++	++	++	+++	+++	+++	+++
	Mus	ID	ID	ID	ID	ID	ID	++	ID	++	ID	++	ID	++
ERβ	Epi	–	–	–	–	–	–	–	–	–	–	–	–	–
	Str	–	–	–	–	–	–	–	–	–	–	–	–	–
	Mus	ID	ID	ID	ID	ID	ID	–	ID	–	ID	–	ID	–
PRs	Epi	±	±	±	±	±	±[c]	++	±[c]	++	±[c]	+++	±[c]	+++
	Str	–	–	–	±	±	±	–	+	–	+	±	+	+
	Mus	ID	ID	ID	ID	ID	ID	–	ID	±	ID	++	ID	++
AR	Epi	–	–	–	±	±	±	±	±	±	±	±	±	±
	Str	–	–	–	±	±	±	±	±	±	±	±	±	±
	Mus	ID	ID	ID	ID	ID	ID	±	ID	±	ID	+	ID	+

[a]: Data from Okada *et al.* (2003)

Epi: epithelial cells, Str: stromal cells, Mus: muscle cells.

INF: infundibulum, AMP: ampulla, IST: isthmus, UTJ: uterotubal junction.

+++: marked, ++: moderate, +: weak, ±: slight, –: negative, ID: indistinguishable.

[b]: Some epithelial cells stained negative. [c]: Some epithelial cells stained negative or moderately.

for β-tubulin IV, but positive for ERα.

During neonatal development of the uterus and vagina

Region-specific expression of ER was previously reported in the female reproductive tracts of neonatal mice. On the day of birth, ER was expressed in epithelial cells of the oviduct, cervix and vagina, but not in the uterus. In contrast, stromal cells expressed ER in all of these organs, including the uterus (Yamashita *et al.*, 1989; Sato *et al.*, 1992, 1996; Ohta *et al.*, 1993, 1996; Li, 1994; Fishman *et al.*, 1996). The onset of uterine epithelial ER immunostaining was ND 4 in mice (Yamashita *et al.*, 1989) and ND 5 in rats (Table 3, Ohta *et al.*, 1996). ER mRNA was observed starting on ND 7 in rats (Fishman *et al.*, 1996). However, strain differences in the ontogenetic localization of ER were reported in uterine epithelial cells of neonatal BALB/c and CD-1 mice (Bigsby *et al.*, 1990). Mowa and Iwanaga (2000b) evaluated ERα expression in the neonatal female rat reproductive tract using *in situ* hybridization. No ERα mRNA signals were found in the uterine, cervical or vaginal epithelia until ND 4-6. The first appearance of ERα mRNA signals in uterine and vaginal epithelia was recognized by ND 4 and 6, respectively. In the vagina, the strongest and most distinct signals for ERα mRNA were localized in the basal layer of the epithelium and diminished in strength towards the lumen. Uterine glandular epithelium expressed signals for ERα mRNA by ND 14. The cervix was the only portion of the developing reproductive tract lacking epithelial ERα. Jefferson *et al.* (2000) also revealed postnatal ERα and ERβ expression in the female mouse reproductive tract using RPA and immunohistochemistry. The results of RPA revealed that the expression of ERα mRNA in the uterus was found at all stages of neonatal development, but no ERβ mRNA appeared until ND 26. Moreover, in immunohistochemistry, ERα was detected in stromal cells of the uterus throughout the postnatal period and in epithelial cells as early as ND 5. Similarly, ERα immunostaining was detected on ND 5 in mouse uterine epithelium, and at birth in both the Müllerian and sinus vagina (Kurita *et al.*, 2001). Signals for ERβ mRNA were only weakly expressed in the reproductive tract during the rat postnatal period (Mowa and Iwanaga, 2000b), and there was no expression of ERβ protein in the mouse uterus (Jefferson *et al.*, 2000).

ONTOGENIC EXPRESSION OF PROGESTERONE RECEPTORS

During prenatal development of the Müllerian duct

In adult rodents, expression of PR has been reported in both the epithelium and the stroma of the uterus and vagina (Ohta *et al.*, 1993, 1996; Graham and Clarke, 1997; Mowa and Iwanaga, 2000a; Shughrue *et al.*, 1998; Wang *et al.*, 1999). Developmental changes in PR have been reported in the uterus of guinea pigs and rats using binding assay with radio-labeled ligand (Pasqualini and Nguyen, 1980; Nguyen *et al.*, 1988), showing a developmental increase in P4 binding sites. In the RT-PCR study, PR mRNA expression was detected from 15.5 dpc and increased gradually by 21.5 dpc in the rat fetus (Okada *et al.*, 2002b). Significant increases were exhibited on 21.5 dpc compared to 15.5 dpc and 17.5 dpc. Localization of PR protein was assessed during rat development by immunohistochemistry using a monoclonal antibody against both PR-A and PR-B (PR-A+B) (Table 4). PR-A+B immunostaining was localized within the nuclei of the Müllerian epithelium. Although the vagina had not yet been formed by 15.5 dpc, slight epithelial PR staining could be detected in the oviduct and uterus by this time. Weak staining of Müllerian epithelial PR appeared in the upper vaginal region by 17.5 dpc. The intensity of Müllerian epithelial PR staining increased gradually from 15.5 dpc until 19.5 dpc within all three of the regions. Staining levels became constant by 19.5 dpc and moderate staining appeared through 21.5 dpc. In contrast, mesenchymal PR staining was faint in all regions of the Müllerian duct throughout gestational development (Okada *et al.*, 2002b). Kurita *et al.* (2001) also reported negative immunostaining for PR-A+B in mesenchymal cells of the uterus, upper vagina, and lower vagina during prenatal development in mice. In order to investigate the differential expression of PR isoform mRNAs in the oviduct, two distinct primer pairs were used; one detected A and B isoforms equally and the other was specific for B isoform (Okada *et al.*, 2003). Oviductal PR-A+B and PR-B were equally expressed at low levels from 15 dpc until birth.

During neonatal development of the oviduct

Neonatal development of oviductal epithelial cells is regulated by E2 and P4 (Abe and Oikawa, 1993). Neonatal PR-A+B ontogeny in oviduct has been reported in mice (Li, 1994) and rats (Okada *et al.*, 2003). Li (1994) demonstrated that in mice, staining of the epithelial PR was weak to positive from ND 1 to 3,

Table 3. Ontogenetic immunolocalization of ERα, and PR-A+B in the neonatal female rat uterus[a].

	Cell type	Neonatal days												
		0	1	2	3	4	5	7	10	12	15	20	25	30
ERα	Epi	–	–	–	–	–	–/+	+/++	++	++	++	++	++	+++
	Str	–	+/++	++	++	++	++	++	+	++	++	++	++	++
	Mus	ID	ID	ID	ID	ID	++	++	+	++	++	++	++	++
PRs	Epi	–	–	–	–	–	–/+	+/++	++	++	+++	+++	+++	+++
	Str	–	–	–	–	–	–	–	–	–/+	++	++	++	++
	Mus	ID	ID	ID	ID	ID	–	–	–	–	–/+	+	++	++

[a]: Data from Ohta *et al.* (1996)

Epi: epithelial cells, Str: stromal cells, Mus: muscle cells.

+++: *marked*, ++: *moderate*, +: *weak*, –: *negative*, ID: *indistinguishable*.

and moderately positive from ND 7, stromal and muscular PR was evident from ND 1. In our rat study, PR immunoexpression was evident in epithelial cells at slight levels at birth, however, it was not present in stromal cells (Table 2). Slight epithelial PR expression continued until ND 5 in the undifferentiated oviduct, and until ND 20 in the INF/AMP region. However, some epithelial cells showed a negative or moderate PR signal in the differentiated INF/AMP region from ND 7 to 20. A double immunohistochemical study on PR-A+B with β-tubulin IV revealed that PR-A+B is expressed specifically in ciliated epithelial cells, but not in nonciliated cells, the same as ERα (Okada *et al.*, 2003). In contrast, epithelial PR staining was intense in the differentiated IST/UTJ region, and showed moderate and marked signals on ND 7 to 10, and ND 15 to 20, respectively. PR was also detected in stromal cells after ND 3, but was absent from the IST/UTJ region on ND 7 to 10. Muscle cells showed slight and moderate PR staining in the IST/UTJ region on ND 10, and from ND 15 to 20, respectively. Oviductal PR-A+B and PR-B mRNAs were equally expressed at low levels from birth to ND 3, and both increased gradually from 15 dpc to ND 5. PR-A+B then increased markedly until ND 20, but PR-B expression continued to increase moderately from ND 7 to 20, resulting in a decrease in the percentage of PR-B to one tenth vs. one quarter against PR-A+B (Okada *et al.*, 2003). At present, although little evidence has been available to define the physiological significance of P4 action via PR-A and/or PR-B, it seems highly probable that differential expression of the two isoforms in the oviduct is fundamental for cell growth, differentiation, and function in response to P4. PR-A is reportedly able to act as a transcriptional inhibitor of PR-B when both proteins are co-expressed (Vegeto *et al.*, 1993). Gava *et al.* (2004) described the differential expression of PR-A and PR-B between cell types in the cycling mouse oviduct. PR-A was the predominant isoform in the oviduct, observed within both epithelial and stromal cells of the AMP and IST regions. In contrast to PR-A expression, PR-B was detectable only in epithelial cells lining the AMP and IST regions.

During neonatal development of the uterus and vagina

Immunohistochemical uterine and vaginal PR expression has been described in mice (Li, 1994; Kurita *et al.*, 2001) and rats (Ohta *et al.*, 1993, 1996). Li (1994) detected positive PR staining in uterine epithelial cells at birth. However, other reports failed to detect PR at birth, but first detected it on ND 3 in mice and ND 5 in rats (Table 3, Ohta *et al.*, 1996). Uterine epithelial PR increased with subsequent postnatal development in both mice and rats. Similarly, uterine stromal and muscular cells expressed PR from birth in mice, but not in rats. Rat stromal and muscle PR was found in the uterus from ND 12 and ND 15, respectively. Kurita *et al.* (2001) evaluated vaginal PR expression during postnatal development in mice, but epithelial immunostaining for PR was negative from birth to ND 14 in both the Müllerian and sinus vagina. No report on isoform expression for uterine or vaginal PR during postnatal development has been found.

ONTOGENIC EXPRESSION OF ANDROGEN RECEPTOR

In rat uterus, AR has been identified by both immunohistochemistry and *in situ* hybridization in epithelial, stromal, and smooth muscle cells (Hirai *et al.*, 1994). In human vagina, AR immunoreactivity was found in both epithelial and stromal cells (Hodgins *et al.*, 1998; Pelletier *et al.*, 2000). AR was also expressed

Table 4. Ontogenetic immunolocalization of PR-A+B in the fetal female rat reproductive tract[a].

Tissue	Cell type	Prenatal days			
		15	17.5	19.5	21.5
Oviduct	Epithelium	±	±	+	+
	Mesenchyme	–	–	–	–
Uterus	Epithelium	±	±	+	+
	Mesenchyme	–	–	–	–
Upper vagina	Epithelium	NF	±	+	+
	Mesenchyme	NF	–	–	–

[a]: Data from Okada *et al.* (2002b)
+: moderate, ±: slight, –: negative, NF: Not formed.

in the female reproductive tracts of rat fetuses. Although AR levels were similar at 17 dpc in the urogenital tubercle of male and female fetuses, the amount of AR decreased after 18 dpc in female urogenital tissues (Bentvelsen *et al.*, 1994). AR mRNA was also expressed in the rat oviduct, and increased gradually with development of the pre- and neonatal rats (Okada *et al.*, 2003). Drews *et al.* (2001) investigated the immunohistochemical localization of AR in the Müllerian duct of fetuses at 17 dpc and 18 dpc. AR staining showed negative throughout the duct except for mesenchymal cells in the sinus region. In the developing oviduct, no AR immunoreactivity was detected on 15 dpc and 19 dpc (Table 2, Okada *et al.*, 2003). Both epithelial and stromal ARs were first detected at a low level on ND 3, and maintained at similar level until ND 20 in all oviduct regions. In the IST and UTJ, muscle AR was observed at slight and weak levels from ND 7 to 10, and ND 15 to 20, respectively. AR was predominantly detected in nonciliated epithelial cells in the INF/AMP, and stromal and muscle cells in the rat oviduct. Recently, a direct inhibitory effect of the ERα/AR heterodimer on both ERα and AR transactivational properties has been reported (Panet-Raymond *et al.*, 2000). Although, in the rat oviduct, co-localization of AR and ERα has not been determined, both AR and ERα were expressed in stromal, muscle, and nonciliated epithelial cells. Interaction of AR and ERα, therefore, may possibly occur in the rat oviduct as well as in the uterus.

FINALLY

Xenoestrogens or endocrine disruptors bind ER and may cause estrogenic effects in humans. In laboratory rodents, perinatal exposure to estrogenic chemicals causes a variety of abnormalities in the female reproductive tract. Thus rats and mice have been used as a model system for the investigation of the perinatal effects of estrogenic chemicals to human embryos and fetuses. Meanwhile, several subtypes and isoforms of sex steroid hormone receptors, including ER, have been identified, and understanding the interactions between these subtypes and isoforms can shed light on the molecular mechanism of hormone action. In order to clarify the effect of estrogenic chemicals on the reproductive tract, it is essential to understand in which organs, on which cell types, and during what stages of development these chemicals can act. Accumulating evidence suggests that different receptor subtypes and isoforms showed developmental stage-, organ- and cell type-specific expression patterns in the female reproductive tract, demonstrating the importance of finding which are target organs and cells for each developmental stage. In addition, we need to elucidate the molecular mechanisms of estrogenic chemicals. Microarray methodology will provide a powerful technique for identifying global gene expression changes during development and for understanding the molecular basis of the adverse effects of the estrogenic chemicals that can alter signaling systems (Watanabe *et al.*, 2002, 2003, 2004; Wu *et al.*, 2003; Hu *et al.*, 2004). It is necessary to study targets of estrogenic chemicals with consideration to the developmental stage and with an understanding of the phenotypes that result from perinatal exposure, in addition to the changes in gene expression obtained from microarray and other molecular techniques.

ACKNOWLEDGMENT

This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (A) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, a Research Grant from the Ministry of Environment, and a Health Sciences Research Grant from the Ministry of Health, Labour, and Welfare, Japan.

REFERENCES

Abe, H. and Oikawa, T. (1993): Effects of estradiol and progesterone on the cytodifferentiation of epithelial cells in the oviduct of the newborn golden hamster. Anat. Rec., **235**, 390-398.

Armstrong, D.T., Moon, Y.S. and Leung, P.C. (1976): Uterotrophic effects of testosterone and 5α-dihydrotestosterone in intact and ovariectomized immature female rats. Biol. Reprod., **15**, 107-114.

Bentvelsen, F.M., McPhaul, M.J., Wilson, J.D. and George, F.W. (1994): The androgen receptor of the urogenital tract of the fetal rat is regulated by androgen. Mol. Cell. Endocrinol., **105**, 21-26.

Bigsby, R.M., Aixin, L., Luo, K. and Cunha, G.R. (1990): Strain differences in the ontogeny of estrogen receptors in murine uterine epithelium. Endocrinology, **126**, 2592-2596.

Byskov, A.G. and Høyer, P.E. (1994): Embryology of mammalian gonads and duct. In The Physiology of Reproduction, 2nd ed. (Knobil, E. and Neill,