Developmental changes of the oestrogen receptor-α and -β mRNAs in the female reproductive organ of the rat – an analysis by in situ hybridization

C N Mowa and T Iwanaga
Laboratory of Anatomy, Graduate School of Veterinary Medicine, Hokkaido University, Kitaku, Sapporo 060-0818, Japan

Abstract
This study employed an in situ hybridization technique to compare the cellular expression of oestrogen receptor (ER) subtypes, ERα and β, in the female reproductive organ of the rat during prenatal and postnatal periods. Diffuse signals of ERα and β mRNAs were co-expressed in the foetal ovary; they were weak and inconsistent before onset of gonadal differentiation, but increased in intensity with age. ERβ mRNA signals in the ovary sharply increased in intensity to adult levels by postnatal days 6–7, whereas those of ERα mRNA remained unchanged after birth. ERα was the sole subtype expressed during the prenatal period from the oviduct to the vagina, being localized mainly to the sub-epithelial stromal cells, and remained predominant thereafter. Signals for ERα mRNA in the epithelia were confined to the oviduct during prenatal and early postnatal periods; those in uterine and vaginal epithelia first appeared by postnatal days 4–5 and 6 respectively. Expressions of ERβ mRNA in the reproductive tract were absent during the prenatal period, and were weakly expressed during the postnatal period. Thus, oestrogen action in the developing ovary may be co-mediated by both ERα and β, whereas ERα may be the primary mediator in the differentiation and growth of the female reproductive tract.


Introduction
Although factors regulating the developmental differentiation and growth of the female reproductive organ are not well known, oestrogen has been implicated as one such factor (Braham et al. 1985, Braham & Sheeham 1995). In order to better understand the involvement of oestrogen in both the normal and pathological development of the reproductive organ, it is necessary to determine the distribution and expression pattern of oestrogen receptors (ERs). Furthermore, experimental data indicating that exposure to exogenous oestrogens during development induces abnormalities in the growth and differentiation of reproductive organs (Iguchi et al. 1988, Medlock et al. 1988, Newbold et al. 1998) has generated considerable interest in the role of oestrogen during ontogeny. A clear understanding of the cellular distribution of ER in the reproductive organ during development will help elucidate the role of oestrogen and the effects of prenatal exposure to environmental oestrogens on reproduction and fertility.

Oestrogenic effects are believed to be mediated principally by ERα and ERβ (Koike et al. 1987, Kuiper et al. 1996, Mosselman et al. 1996). ER is detectable in mammalian tissues beginning with the unfertilized oocyte through adulthood, with the exception of a brief morula stage (Holderegger & Keefer 1986, Wu et al. 1992, Hou & Gorski 1993, Kuiper et al. 1996). After the appearance of the reproductive organ, around foetal days 12–13, ER expression becomes concentrated in the reproductive organ (Hou & Gorski 1993). Ontogenetical studies on ER expression in the rodent reproductive organ have mostly been limited to reports of oestrogen binding assays (Clark & Gorski 1972, Kuiper et al. 1997), autoradiography (Cunha et al. 1982, Holderegger & Keefer 1986), immunostaining (Greco et al. 1991) and RT-PCR (Wu et al. 1992, Hou & Gorski 1993, Gorski & Hou 1995). Data on the expression of ER mRNA in specific cell types of female reproductive tissue compartments are largely confined to the adult (Shughrue et al. 1998, Mowa & Iwanaga 2000), with minimal postnatal reports (Fishman et al. 1996), and, to the best of our knowledge, none for the prenatal period.

This report contains the expression patterns of ERα and -β mRNAs in the reproductive organ of the female rat during prenatal development, and further extends the findings of previous studies on the postnatal period.
Materials and Methods

Animals

Immature female Wistar rats used in this study were taken from foetal day 12 to postnatal day 24 (except foetal days 15 and 18; postnatal days 8, 10, 13, 15, 17–19, 21–23) \( (n=62, \) approximately three animals per age group). The rats were sacrificed and sampled at the above ages. Gender was ascertained histologically by confirming the gonadal sex.

Probes

Several non-overlapping antisense oligonucleotide probes (45 mer in length) specific to either ER\( \alpha \) or \( \beta \) mRNA were used initially in order to optimize sensitivity. The selected probes for ER\( \alpha \) and \( \beta \) were complementary to 301–346 bp of ER\( \alpha \) cDNA (Accession no. Y00102) (Koike et al. 1987) and 45–90 bp of ER\( \beta \) cDNA (Accession no. U57439) (Kuiper et al. 1996) respectively. The oligonucleotides were labelled with \(^{35}\)S-dATP, using terminal deoxyribonucleotidyl transferase (Promega, Madison, WI, USA) at a specific activity of \( 5 \times 10^{8} \) d.p.m./\( \mu \)g DNA.

In situ hybridization

The in situ hybridization procedure was performed as previously described (Mowa & Iwanaga 2000). Briefly, 20 \( \mu \)m cryostat sections obtained from frozen fresh tissues were mounted on glass slides pre-coated with 3-aminopropyltriethoxysilane, fixed in 4\% paraformaldehyde for 10 min, and then acetylated with 0\%25 acetic anhydride in 0\%1 M triethanolamine-HCl (pH 8.0) for 10 min.

The prepared sections were pre-hybridized for 2 h in a buffer containing 50\% formamide, 0\%1 M Tris–HCl (7.5), 4\% SSC (1 \( \times \) SSC; 150 mM NaCl and 15 mM sodium citrate), 0\%02% Ficoll, 0\%02% polyvinylpyrrolidone, 0\%02% BSA, 0\%6 M NaCl, 0\%25 sodium dodecyl sulphate (SDS), 200 \( \mu \)g/ml tRNA, 1 mM EDTA and 10\% dextran sulphate. Hybridization was performed at 42\%C for 10 h in pre-hybridization buffer supplemented with 10000 c.p.m./\( \mu \)l of \(^{35}\)S-labeled oligonucleotide probes. The slides were washed at room temperature for 20 min in 2 \( \times \) SSC containing 0\%1\% sarkosyl (Nacalai tesque, Kyoto, Japan) and twice at 55\%C for 40 min in 0\%1 \( \times \) SSC containing 0\%1\% sarkosyl. The sections were either exposed to Hyperfilm-\( \beta \)-max (Amersham, Buckinghamshire, UK) for 2 weeks, or dipped in Kodak NTB2 nuclear track emulsion (New York, NY, USA) and exposed for 4–8 weeks.

The specificity of the in situ hybridization was confirmed by the disappearance of signals when an excess dose of corresponding cold oligonucleotides was added to the hybridization fluid. Consistent ER mRNA signals above background levels were considered positive and scored as strong, moderate, weak, or very weak.

Results

Table 1 summarizes the chronological order and the cellular expression patterns of ER\( \alpha \) and \( \beta \) mRNAs during development in the rat female reproductive organ.

Ovary

Initial signals, just above background levels, for both ER\( \alpha \) and \( \beta \) mRNA were detected in the undifferentiated gonad by foetal day 14 and increased in intensity and consistency thereafter. Concomitant with their appearance by about postnatal day 12, interstitial glands and theca cells exhibited fairly distinct ER\( \alpha \) mRNA signals (Fig. 1D), with a consistent intensity until postnatal day 24, the last day examined. Granulosa cells lacked ER\( \alpha \) mRNA expression throughout the period of study (Fig. 1E). It is worth noting that the germinal epithelium expressed detectable signals of ER\( \alpha \) mRNA by foetal day 17 and increased in strength with age thereafter.

The first appearance of the signals and expression pattern of ER\( \beta \) mRNA in the ovary during the prenatal period were similar to those of ER\( \alpha \) mRNA; the signals were detected on foetal day 14 in the undifferentiated gonad, and increased in intensity with advancing age (Fig. 1A, B, C). However, during the neonatal period, both the distribution and signal intensity of the ER subtypes diverged. By postnatal days 4–6, ER\( \beta \) mRNA signals in the ovary had sharply increased in intensity and were concentrated in the granulosa cells, attaining adult levels of intensity by postnatal days 6–7 (Fig. 1B).

Reproductive tract

The first significant signals for ER\( \alpha \) mRNA in the reproductive tract were detected by foetal day 14, primarily in mesenchymal cells located around tube-like ducts, which were closely associated with the ovary (Fig. 2A). These tubular structures were identified as the Müllerian ducts from which different regions of the female reproductive ducts, such as the oviduct, uterus, cervix and cranial vagina, differentiate. By foetal days 16–17, various intensities of ER\( \alpha \) mRNA signals were found in the mesenchyme of the different regions of the reproductive tract, following their differentiation from the Müllerian ducts (Fig. 2B–E). The cervical and vaginal mesenchyme displayed the most intense signals of ER\( \alpha \) mRNA by foetal days 18–19 (Fig. 2D, E). However, sub-epithelial mesenchymal cells of the cervix had lower signal intensity (Fig. 2D).

By foetal day 17, consistent ER\( \alpha \) mRNA signals were detectable in the precursor epithelial cells of the oviduct (Fig. 2C), but not in the epithelia of the remaining reproductive tract; no ER\( \alpha \) mRNA signals were found in uterine, cervical or vaginal epithelia until postnatal days 4–6 (Fig. 2B, D, E). By postnatal day 4, the intensity of
ERα mRNA expression in oviductal epithelium became comparable to adult levels (Fig. 2G). The oviduct was, therefore, the only region with significant signals of epithelial ERα mRNA during prenatal and early neonatal periods. The first appearance of ERα mRNA signals in uterine and vaginal epithelia were recognizable by postnatal days 4 and 6 respectively (Fig. 2F). 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 postnatal day 14 (Fig. 2H). The cervix was the only portion of the developing reproductive tract lacking epithelial ERα mRNA signals throughout the developmental period examined in this study.

Signals for ERβ mRNA were not detectable in the reproductive tract during the prenatal stage and were only weakly expressed during the postnatal period (Fig. 2I).

**Discussion**

Recent conclusive findings that ERα- and ERαβ-knockout mice exhibit normal development of the reproductive organs (Couse & Korach 1999, Couse et al. 1999) have led to the need to verify the roles of ER in organogenesis. The absence of any gross phenotypes in the ER knockout mice has cast doubts on the involvement of oestrogen in development of the reproductive organ. It is, however, interesting to note that the onset of ER mRNA expression in the foetal reproductive organ, as reported here, is extremely precocious, virtually coinciding with the initial appearance of the reproductive organ. Furthermore, exposure to exogenous oestrogen during development induces abnormalities in both growth and differentiation of the genital organs in the rat (Medlock et al. 1988).

This present study describes the cellular distribution and chronological order of appearance of both ERα and β mRNAs in the female reproductive organ of the rat during the perinatal period. The broad distribution of the ERα mRNA signals in the reproductive organ, in contrast to the gonad-restricted expression of ERβ mRNA, indicates that ERα may be the predominant mediator of oestrogen-induced effects during development, and may be involved in inducing the reported detrimental reproductive abnormalities arising from exposure to exogenous oestrogens during ontogeny (Medlock et al. 1988). However, oestrogen effects mediated by ERβ are largely confined to ovarian development and, possibly, to abnormalities which arise when exposed to endocrine disruptors (Newbold et al. 1983). The differential distribution patterns of ERα and ERβ mRNAs during development reported here is consistent with data from previous in situ hybridization studies of the adult female rat (Shughrue et al. 1998, Mowa & Iwanaga 2000). However, hybridization signals for ERα...
mRNA during the prenatal period are absent from epithelial cells, unlike in the adult female reproductive tract, and are primarily confined to the stromal cells, except in the oviduct where significant expressions of ERβ mRNA in the epithelium appeared by as early as foetal day 17. We have presented for the first time the cellular distribution of ERβ mRNA in the rat ovary during prenatal and early postnatal periods and extended the study of ERα mRNA expression during foetal and neonatal development. Since the ERβ mRNA probe used in this study was picked from the A/B domain, it was expected to detect both ERβ1 and β2 isoforms, which chiefly differ in the ligand domain (Paterson et al. 1998). The present data was consistent with the results of several earlier studies which studied ER distribution by immunohistochemistry using perinatal tissues of mice (Yamashita et al. 1989, Greco et al. 1991, Jefferson et al. 2000) and rats (Sar & Welsch 1999), binding assay using postnatal rats (Clark & Gorski 1972) and in situ hybridization for ERα using postnatal rats (Fishman et al. 1996). There are, however, a few discrepancies between our findings and some immunohistochemical reports. In the present study, ERβ mRNA signals were first detected in the ovary by foetal day 14, in contrast to Sar and Welsch (1999), who could only detect immunoreactivity for ERβ protein in the rat ovary by postnatal day 5. It is possible that the different findings between their study and the present one could be due to differences in sensitivity between the methods used. Another possible cause of discrepancy may be attributed to the fact that transcription of mRNA precedes protein translation.

In the rat, although the ovary becomes discernible by foetal days 14–15, it is not until around postnatal day 3 that folliculogenesis commences (Malamed et al. 1992). The co-expression of both ER subtypes with comparatively intense signals in the foetal ovary suggests their role in the initial stage of ovarian development. The onset of folliculogenesis is concomitant with detection of basal oestrogen levels in the blood, which significantly increases with age (Weniger et al. 1993). Thus, the sharp rise of ERβ mRNA signals in granulosa cells in the first week of the neonatal period, coincident with the onset of folliculogenesis,
indicates its involvement in follicular differentiation and function. This conclusion is consistent with that of Drummond et al. (1999) using rats, who noticed a marked rise in both isoforms of ERβ mRNA, particularly between postnatal days 8 and 12 (Paterson et al. 1998). Localization of ERα mRNAs in ovarian stromal cells, and later in the

Figure 2  Distribution of ERα (A–H) and β (I) mRNA in the female reproductive tract of the rat during the perinatal period. (A) Bright-field (I) and dark-field (II) images demonstrate weak signals of ERα in stromal cells (s) of the Müllerian duct at foetal day 14, concentrated largely on the edges (*). e=epithelium. (B) Distinct ERα signals are visible in the stroma but not epithelium (e) of uterus at foetal day 17. (C) In the oviduct, both the stroma and epithelium express clear ERα signals at foetal day 19. (D) The cervix at foetal day 19 shows pronounced ERα in the stroma but not in the subepithelial stroma (se). (E) The vagina at foetal day 19 expresses intense ERα signals in the subepithelial stroma but virtually none in the epithelium. (F) The vagina at postnatal day 6 exhibits pronounced signals of ERα mRNA in the stroma and basal half of the epithelium (be) (G) Oviductal epithelium demonstrates intense signals at postnatal day 4 (o, ovary). (H) In the uterus, signals for ERα are distinctly expressed in the glandular epithelium (ge) at postnatal day 14. (I) ERβ are exhibited in the stroma of the uterus at postnatal day 7, as revealed by the bright-field (i) and dark-field (ii) images. Bars = 100 μm (B, F–H), 200 μm (A, C–E) and 312 μm (I).
theca and interstitial glands, however, suggests its role in mediating their growth, differentiation and function, such as steroidogenesis.

The exact ligand acting on ER in the female reproductive organ of the prenatal animals is a matter of speculation. Since expression and activity of aromatase enzyme, a critical steroidogenic enzyme required for oestrogen synthesis, has been demonstrated in some studies of the rodent foetal ovary, foetal oestrogen may be a candidate (Picon et al. 1988). However, other studies failed to consistently detect three of the four essential steroidogenic enzymes in the rat foetal ovary (including the crucial P450 aromatase) (Greco & Payne 1994), implying that the foetal ovary is incapable of producing any significant levels of oestrogen. Serum levels of oestrogen in the rat significantly increase only around postnatal day 9 (Dohler & Wutkue 1975). Oestrogen has co-regulators such as epidermal growth factors, which are able to mimic the effects of oestrogen (Ignar-Trowbridge et al. 1992, Curtis et al. 1996). It is possible, therefore, that these oestrogen co-regulators may act as ‘ligands’ for ER during ontogeny, solely or synergistically with foetal oestrogen, at a time when foetal oestrogen levels are functionally low or absent. Additionally, maternal oestrogen, which increases in serum with advancing gestational age and can cross into foetal blood circulation (Habert & Picon 1984), may also act on foetal ER.

The female reproductive tract has long been known to be a classic target of oestrogen action in mammals (Braham et al. 1985). Consistent with past autoradiographic (Holderegger & Keefer 1986) and immunohistochemical studies (Gorski & Hou 1995), which suggest the existence of ER in the mesenchyme around genital ducts of foetal mice, the present in situ hybridization analysis clearly demonstrates stromal ERα mRNA signals in the developing reproductive tract as early as foetal day 14, prior to differentiation of the reproductive tract. Furthermore, the presence of epithelial ERα mRNA signals in the duct-like tubes confirmed previous histochemical reports which demonstrated ER-like immunoreactivity in the epithelium of the Mullerian duct (Korach et al. 1988). However, the signals observed in this present study were of greater intensity and more consistent in the mesenchymal cells than in the luminal epithelium. As the different portions of the tract became distinguishable, signals for epithelial ERα mRNA were limited to the oviduct by foetal day 17. It is widely accepted that steroid hormones may produce their effects on epithelia via the mesenchyme and that the mesenchyme is essential for epithelial proliferation, morphogenesis and differentiation (Donjacour & Cunha 1991). Tissue recombinant studies have shown that oestrogen-induced proliferation of the uterine epithelium only requires the expression of ERα in the stromal cells, whereas the induction of epithelial secretory products depends on the presence of ERα in both uterine compartments (Buchanan et al. 1999). Thus, since the epithelia of the foetal uterus, cervix and vagina lacked ERα, mesenchymal ERα may mediate their proliferation and differentiation. The epithelial expression of ERα mRNA in foetuses is in contrast to that of the adult, where epithelia throughout the female reproductive tract expressed intense ERα mRNA signals (Mowa & Iwanaga 2000). The biological significance of the precocious appearance of ERα only in the oviductal epithelium is not known. However, it is interesting to note that the oviduct in the perinatal stage is highly sensitive to detrimental effects of diethylstilbestrol (DES) (Newbold et al. 1983). The lack of ERβ in the foetal and early neonatal female reproductive tract suggests that ERβ, unlike ERα, plays no role in the development of the tract.

References


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