In fetal rat oviduct, ERα mRNA was expressed at least from PD 15, and increased by PD 19. After birth, oviductal ERα mRNA continued to increase until ND 3, and was maintained at a high constant level through to ND 20 (Fig. 2A). Expressions of ERα mRNA during the regular 4-day estrous cycle and early pregnancy are also presented. ERα level was significantly lower at estrus than at other stages ($P<0{\cdot}05$). In the pre-implantation oviduct, ERα was low on GD 0, which were similar to those at estrus, and increased significantly from GD 1 to 3. In the pregnant oviduct, the peak of ERα increase was noted on GD 2 ($P<0{\cdot}01$), while, ERβ mRNA was detected in a low and constant manner as compared with ERα throughout the development and physiological changes examined (Fig. 2A).

For PRs, two distinct primer pairs were used to determine isoform-specific change in the rat oviduct, one detected A and B isoforms equally and the other was specific for B isoform. Oviductal PR-A+B and PR-B were equally expressed at low levels from PD 15 to ND 3, and both increased gradually from PD 15 to ND 5 (Fig. 2B and C). PR-A+B then increased markedly until ND 20, but PR-B expression continued to increase moderately from ND 7 to ND 20, resulting in a decrease in the percentage for PR-B to one-tenth to a quarter against PR-A+B. During the estrous cycle, PR-A+B mRNA level was significantly lower at estrus than at other stages ($P<0{\cdot}05$). However, PR-B was not significantly changed throughout the estrous cycle. In the pre-implantation oviduct, both PR-A+B and PR-B expressions were low on GD 0, and increased from GD 1 to 3. Moreover, the percentage for PR-B against PR-A+B on GD 0 (21·5%) increased to 46·6% on GD 2 (Fig. 2C).

AR mRNA was also expressed in the rat oviduct, and increased gradually with development of the pre- and neonatal rats. However, no change was exhibited in the cycling and pre-implantation rats (Fig. 2A).

Immunohistochemical expressions of ERα and PR-A+B in the prenatal and neonatal rat oviducts

In the uterus, as positive control tissue, ERα and PR-A+B were detected in nuclei of luminal and glandular epithelia, and stroma and muscle layer (Fig. 3) as reported (Ohta *et al.* 1993, Wang *et al.* 2000). With these monoclonal antibodies, ontogeny of immunoexpressions for ERα and PR-A+B in the rat oviduct was determined, and summarized in Table 1. Pre- and neonatal oviducts showed simple tubal structure from PD 15 to ND 5, and subsequently differentiated morphologically into the INF, AMP, IST and UTJ after ND 7. In the prenatal oviduct, immunohistochemical staining for ERα exhibited nuclear signals rated as slight and weak in epithelial cells, and negative and slight in mesenchymal cells on GD 15 and 19 respectively. In the neonatal oviduct, epithelial ERα was exhibited as weak and moderate stainings at birth (ND 0) and from ND 3 to 20 respectively (Fig. 4, left panels). However, some ERα-negative cells were present in the epithelium of the INF/AMP region after ND 10. Stromal cells showed ERα stainings 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 among regions in the oviduct. Moderate ERα staining was also found in muscle cells of the IST/UTJ region from ND 7 to 20. PR-A+B immunoexpression was evident in epithelial cells at slight levels on PD 15 and 19; however, it was not present in mesenchymal cells on either prenatal day (Table 1). Slight epithelial PR-A+B expression continued to ND 5 in the undifferentiated oviduct, and to ND 20 in the INF/AMP region (Fig. 4, middle panels). However, some epithelial cells showed negative or moderate PR-A+B signals in the differentiated INF/AMP region from ND 7 to 20. In contrast, epithelial PR-A+B staining was intense in the differentiated IST/UTJ region, and showed moderate and marked signals on ND 7 and 10, and ND 15 and 20 respectively. PR-A+B was also detected in stromal cells after ND 3, but absent in the IST/UTJ region on ND 7 and 10. Muscle cells showed slight and moderate PR-A+B stainings in the IST/UTJ region on ND 10, and ND 15 and 20 respectively (Table 1).

Immunohistochemical expressions of ERα and PR-A+B in the cycling and pre-implantation oviducts

ERα and PR immunoreactivities were detected in nuclei of epithelial, stromal and muscle cells of all regions in the diestrous oviduct (Fig. 5). Although most of the stromal cells (INF, AMP, IST and UTJ) and muscle cells (IST and UTJ) were positive for

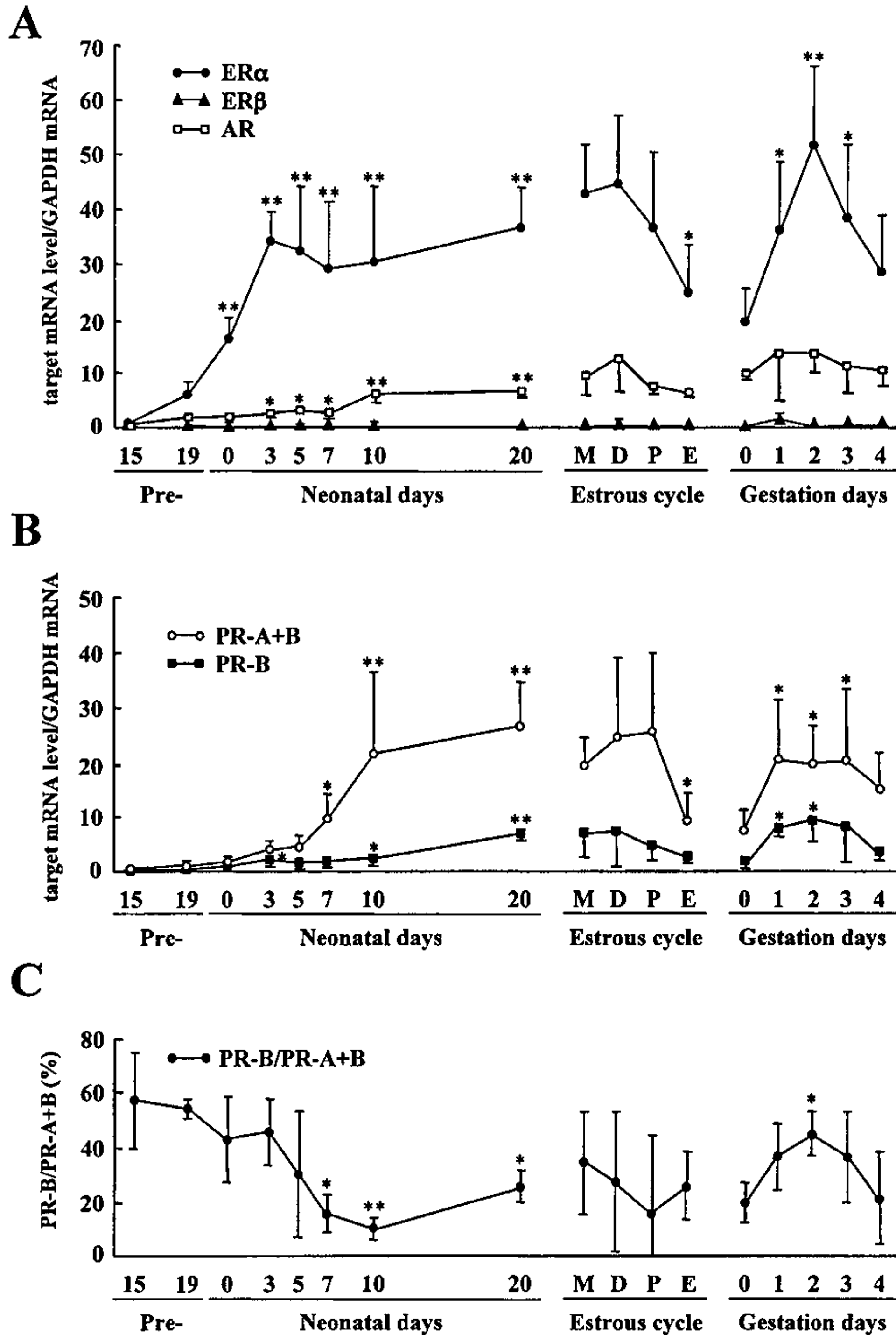

Figure 2 Changes in expressions of ERα, ERβ and AR (A), and PR-A+B and PR-B (B) in the oviduct of the prenatal, neonatal, cycling and pre-implantation rats. Target mRNA expression levels were evaluated by real-time RT-PCR and standardized to GAPDH mRNA expression. Analysis in duplicate was repeated four times in each experiment. Data are represented as means±S.D. from three independent experiments. $^{**}P<0.01$ and $^{*}P<0.05$ vs PD 15, diestrus and GD 0 in developing, cycling and pre-implantation oviduct respectively. Change in the percentage for PR-B against PR-A+B is shown (C).

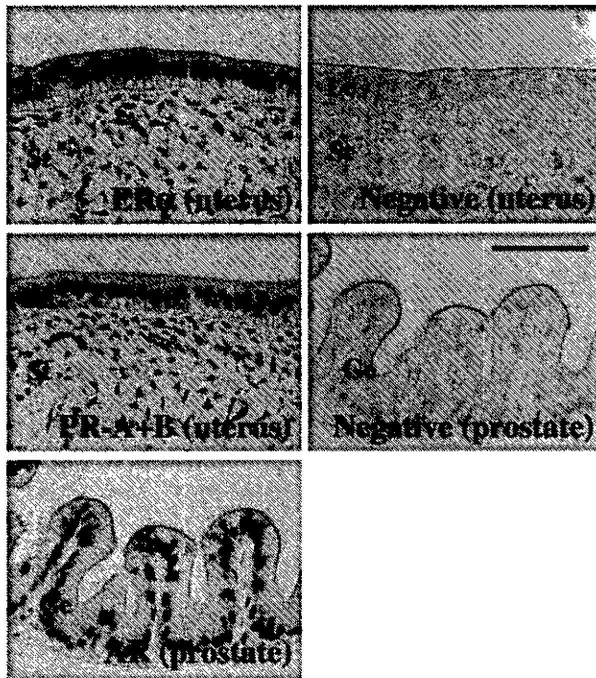

Figure 3 Immunohistochemistry for ERα, PR-A+B and AR in the uterus and prostate of adult rats. ERα and PR-A+B stainings were seen in nuclei of luminal epithelium (Le) and stroma (St) of the uterus. Nuclear staining for AR was detected in glandular epithelium (Ge) of the prostate. ERα and PR-A+B, and AR stainings were abolished by incubation with normal mouse and rabbit IgG respectively (Negative). Bar: 50 μm.

both ERα and PR-A+B, the number of positive epithelial cells varied depending on the oviduct region. Despite positive stainings for ERα and PR-A+B in all epithelial cells of the IST/UTJ, positive stainings were less in those of the INF/AMP (Fig. 5). Staining intensity of ERα was marked in all cell types of all regions. PR-A+B staining intensity was also marked in stromal and muscle cells of all regions, and in epithelial cells of the IST/UTJ, whereas it was weak in epithelial cells of the INF/AMP. PR-A+B staining in epithelial cells was greater in the IST than in the UTJ (Fig. 5).

Although changes in the staining intensity of ERα and PR-A+B in all regions were weak during the estrous cycle and early pregnancy, epithelial, stromal and muscle cells in the IST/UTJ showed apparent changes, the intensity being higher at diestrus and metestrus than at proestrus and estrus (Fig. 6). In the pre-implantation oviduct, immunolocalization of ERα and PR-A+B exhibited a pattern similar to that in the cycling oviducts. Gestational increases in ERα and PR-A+B staining intensities were observed in the IST/UTJ cells (Fig. 6).

Immunohistochemical expression of oviductal ERβ

For immunohistochemistry of ERβ, Weihua *et al.* (2002) recently reported an improved method to detect the ERβ signal in the rat uterus by using urea in place of citrate buffer as the antigen retrieval buffer, and detected apparent nuclear ERβ stainings in rat uterine epithelial and stromal cells. Effectiveness of the antigen retrieval with urea has also been reported for stainings of ERα, PR, AR and other intranuclear antigens in paraffin sections (Taylor *et al.* 1994). Therefore, we employed this method for ERβ immunohistochemistry, and could detect ERβ immunoreactivity in nuclei of uterine luminal and glandular epithelial, and peripheral stromal cells in addition to granulosa cells of the ovary, which served as controls (Fig. 7). To confirm our findings of ERβ protein expression in the rat tissues, we employed two commercially available rabbit polyclonal antisera to rat ERβ as reported (Okada *et al.* 2002*a*): PA1–310 (Affinity Bioreagents, Inc., Golden, CO, USA) and 06–629 (Upstate Biotechnology, Lake Placid, NY, USA) against amino acid residues 467–485 and 54–71 of rat ERβ respectively. With both ERβ antisera, similar results to those with our antibody were obtained in the ovary and uterus by immunohistochemistry using either citrate buffer or urea (data not shown).

In the diestrous oviduct, however, few ERβ-positive cells were found in epithelial cells of the INF/AMP with our (Fig. 7) and two other ERβ antibodies (data not shown). No ERβ was detected in stromal cells of the INF/AMP, or in any cells of the IST/UTJ of the diestrous oviduct. ERβ immunoreactivity was indistinguishable in the pre- and neonatal oviduct (Table 1), and showed no remarkable change during the estrous cycle and pre-implantation period in the adult oviduct (data not shown).

Immunohistochemical expression of oviductal AR

In the adult rat prostate, positive AR was detected in nuclei of glandular epithelial cells (Fig. 3) as previously reported (Husmann *et al.* 1990).

Table 1 Ontogenetic immunolocalization of ERα, ERβ, PR-A+B and AR in the prenatal and neonatal female rat oviduct

		PD		ND										
		15	19	0	3	5	7		10		15		20	
	Cell type						INF/AMP	IST/UTJ	INF/AMP	IST/UTJ	INF/AMP	IST/UTJ	INF/AMP	IST/UTJ
ERα	Epi	±	+	+	++	++	++	++	++[a]	++	++[a]	++	++[a]	++
	Str	–	±	+	++	++	++	++	++	++	+++	+++	+++	+++
	Mus	UD	UD	UD	UD	UD	UD	++	UD	++	UD	++	UD	++
ERβ	Epi	–	–	–	–	–	–	–	–	–	–	–	–	–
	Str	–	–	–	–	–	–	–	–	–	–	–	–	–
	Mus	UD	UD	UD	UD	UD	UD	–	UD	–	UD	–	UD	–
PRs	Epi	±	±	±	±	±	±[b]	++	±[b]	++	±[b]	+++	±[b]	+++
	Str	–	–	–	±	±	±	–	+	–	+	±	+	+
	Mus	UD	UD	UD	UD	UD	UD	–	UD	±	UD	++	UD	++
AR	Epi	–	–	–	±	±	±	±	±	±	±	±	±	±
	Str	–	–	–	±	±	±	±	±	±	±	±	±	±
	Mus	UD	UD	UD	UD	UD	UD	±	UD	±	UD	+	UD	+

PD: prenatal days, ND: neonatal days, Epi: epithelial cells, Str: stromal cells, Mus: muscle cells.
INF: infundibulum, AMP: ampulla, IST: isthmus, UTJ: uterotubal junction.
+++: marked, ++: moderate, +: weak, ±: slight, –: negative, UD: undistinguishable.
[a]Some epithelial cells showed negative stainings. [b]Some epithelial cells showed negative or moderate stainings.

However, in the developing oviduct, no AR immunoreactivity was detected in Müllerian duct cells on PD 15 and 19. Both epithelial and stromal ARs were first detected at a low level on ND 3, and maintained at a similar level until ND 20 in all differentiated regions (Table 1, and Fig. 4, right panels). In the IST and UTJ, muscle AR was observed at slight and weak levels on ND 7 and 10, and ND 15 and 20 respectively.

In the diestrous oviduct, some AR stainings were detected at weak levels in epithelial cells of the INF/AMP (Fig. 5, right panels). In contrast to ERα and PR expressions in all epithelial cells, most epithelial cells did not show AR immunoreactivity in the IST/UTJ. Stainings for stromal and muscle ARs were weak or moderate throughout the oviduct region. However, no remarkable change was observed in the expression and localization of AR during the estrous cycle and early pregnancy.

Immuno-co-localization of epithelial ERα, PR-A+B and AR with β-tubulin IV

To determine surface epithelial cell types expressing ERα, PRs and AR, double immunohistochemical staining for each receptor with β-tubulin IV, a ciliated epithelial cell marker (Renthal *et al.* 1993) was performed. In the INF/AMP, the mucosa formed numerous elaborately branched folds and their lumina were labyrinthine systems of narrow spaces between the branching folds covered with the epithelium. The epithelia were basically simple columnar and consisted of two kinds of cells, ciliated and nonciliated epithelial cells. They sometimes appeared pseudostratified in the oblique sections. The ciliated cells were positive for β-tubulin IV and contained round pale nuclei in the apical or middle region of the cells, while the nonciliated cells were negative for β-tubulin IV and had dark nuclei oval to slender in the basal region. Each expression of ERα, PR-A+B and AR was restrictedly observed in nonciliated epithelial cells, but not in ciliated epithelial cells of the INF/AMP (Fig. 8). In contrast, almost all epithelial cells were negative for β-tubulin IV in the UTJ/IST, but positive for ERα and PR-A+B.

Discussion

Despite extensive research on expression of sex steroid hormone receptors in female reproductive tissues of humans and laboratory rodents, expression, localization and function of these receptors in the oviduct remain unclear. This study demonstrates that ERα, ERβ, PR-A, PR-B and AR are expressed in the rat oviduct throughout physiological conditions in a cell- and region-dependent manner. A lack of these receptors in ciliated epithelial cells of the rat oviductal epithelium was noted.

In the real-time RT-PCR study, expressions of ERα, PR-A+B and PR-B mRNA were detected to be regulated in the fetal, neonatal, cycling and pregnant rat oviduct, suggesting that these receptors may play roles in the physiological functions of the rat oviduct. Oviductal ERβ expression was reported to be low in rats (Saunders *et al.* 1997, Mowa & Iwanaga 2000*a,b*) and mice (Couse *et al.* 1997), while Sar & Welsch (1999) and Jefferson *et al.* (2000) failed to detect ERβ immunopositive cells in oviduct of rats and mice respectively. In the present study, although ERβ mRNA was detected in the rat oviduct by RT-PCR, it was present only at low constant levels throughout physiological conditions. Immunoreactivity for ERβ was limited to a few nonciliated epithelial cells in the INF/AMP. Thus, abundant ERα may be a major ER subtype and play an essential role in development and function of the rat oviduct. On the other hand, two PR isoforms, PR-A and PR-B, are produced from a single gene by transcription at two distinct promoters (Kastner *et al.* 1990), and the ratio of isoforms varies depending on the reproductive tissues during development (Shyamala *et al.* 1990) and estrous cycle (Mangal *et al.* 1997). In the rat oviduct, the present study demonstrated a definite variation in the mRNA expression of PR isoforms evaluated by the ratio of PR-B to total PR during development and different hormonal circumstances as adults. 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 two isoforms in the oviduct is fundamental for the cell growth, differentiation and functions in response to P4. PR-A is reported to be able to act as a transcriptional inhibitor of PR-B when both proteins are co-expressed (Vegeto *et al.* 1993).

In the developing oviduct, ERα, PR-A+B and AR were expressed in both epithelial and mesenchymal or stromal cells, and the staining

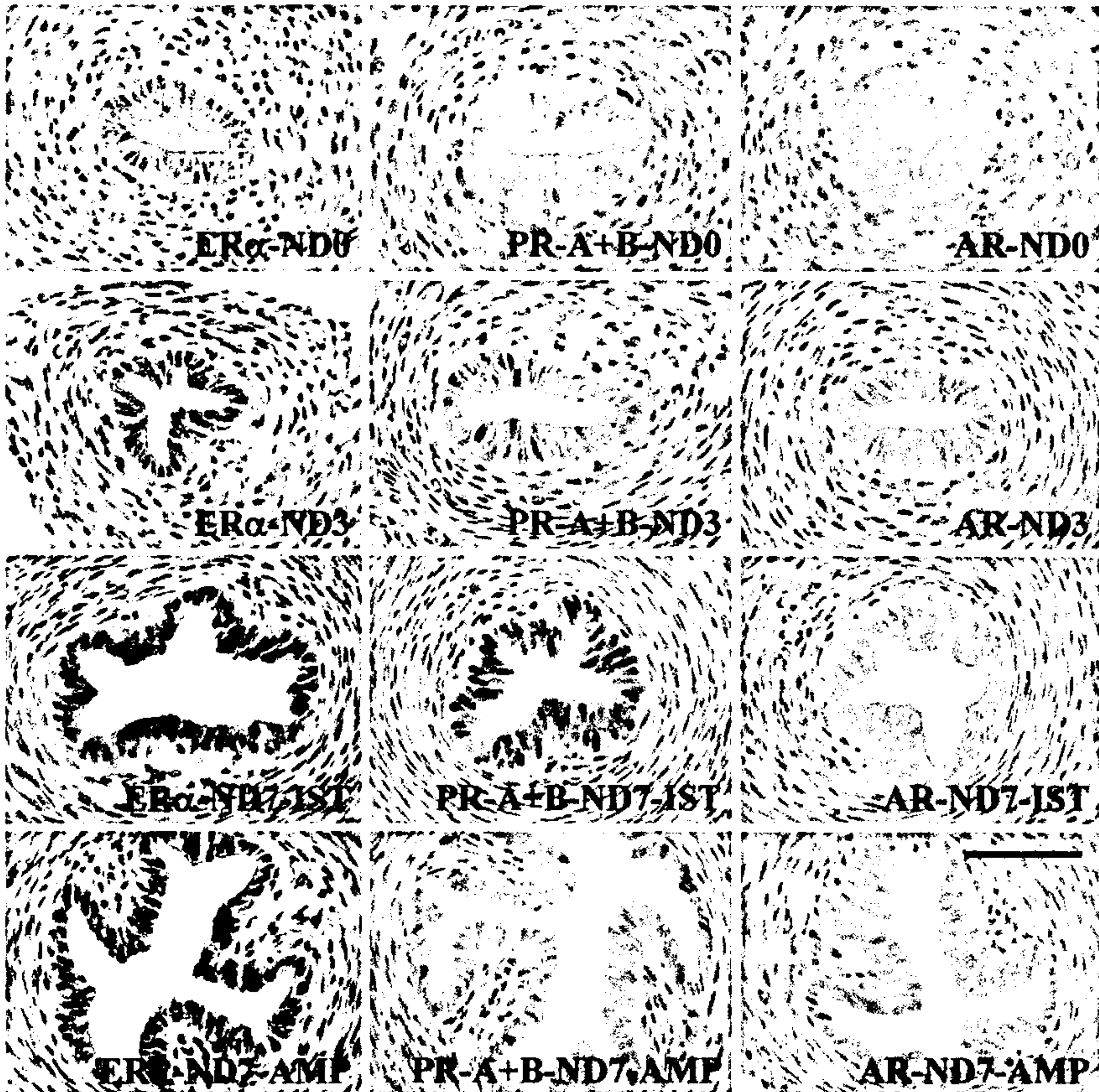

Figure 4 Immunohistochemical localization of ERα, PR-A+B and AR in the developing rat oviduct. ERα (left), PR-A+B (middle) or AR (right) was detected in nuclei of epithelial, stromal and muscle cells of the neonatal rat oviduct. After ND 7, morphological differentiation of the neonatal oviduct occurred. See Table 1 for detail of changes in expressions. All immunoreactivity was abolished by incubation with normal mouse or rabbit IgG as controls (data not shown). Bar: 50 μm.

intensity and mRNA level increased with the growth of neonates. During this early postnatal period when increases in these receptors were observed, immunoreactivity for β-tubulin IV in the rat oviduct appeared between ND 5 and ND 7 (data not shown). Komatsu & Fujita (1978) have reported in their electron-microscopic study that differentiation of ciliated cells, which is believed to be elicited by the initiation of endogenous estrogen (17β-estradiol; E2) production, occurred on ND 5 in the mouse oviduct. Furthermore, neonatal estrogen administration accelerated cilia formation

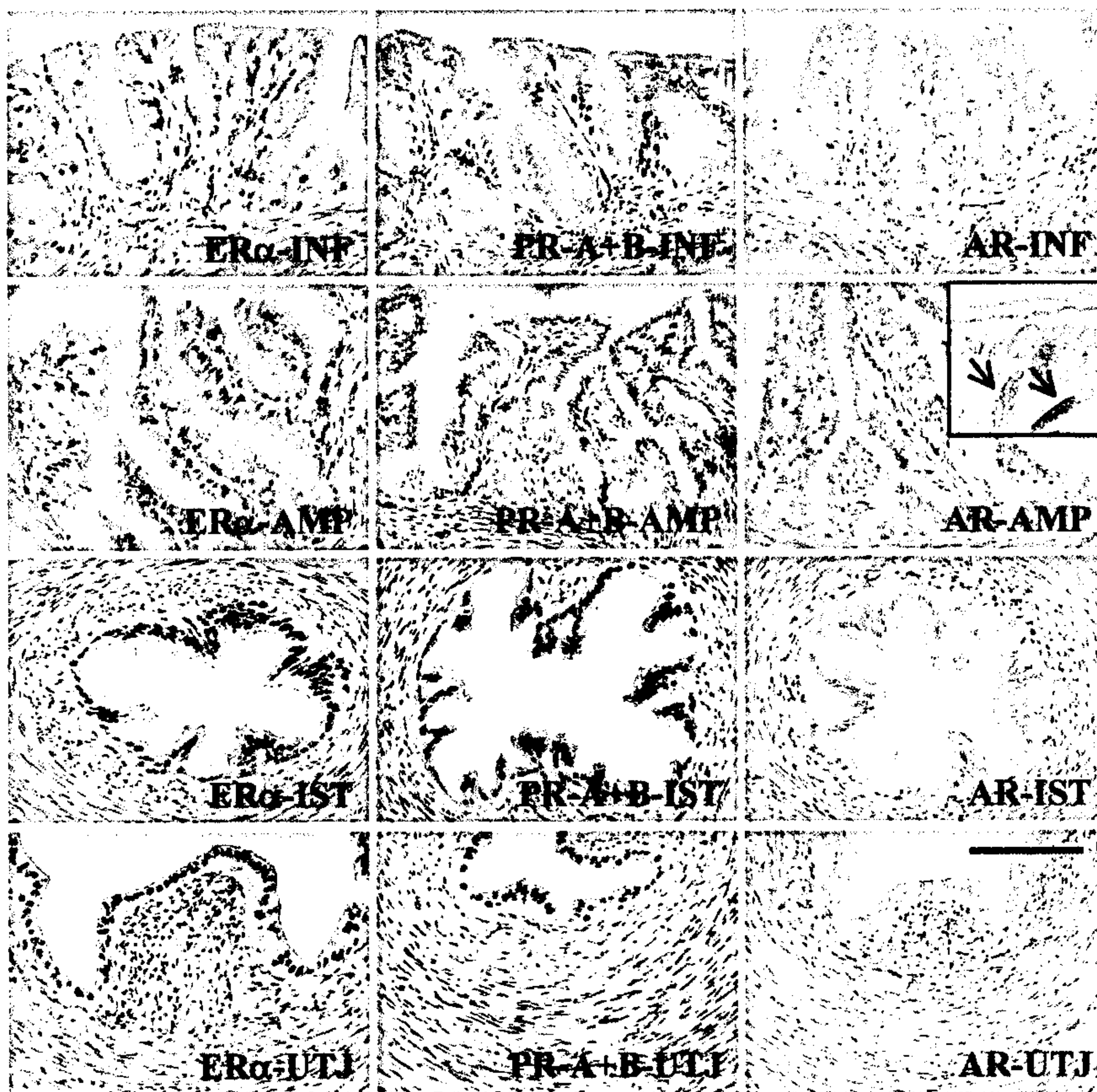

Figure 5 Immunohistochemical localization of ERα, PR-A+B and AR in the diestrous rat oviduct. In the INF and AMP, ERα (left), PR-A+B (middle) and AR (right) were detected in nuclei of epithelial and stromal cells. However, fewer epithelial cells showed immunoreactivity for ERα, PR-A+B and AR. In the IST and UTJ, ERα, PR-A+B and AR were detected in nuclei of epithelial, stromal and muscle cells, except for epithelial AR. Arrows represent high magnification of AR-positive cells, as shown in the inset in AR-AMP. All immunoreactivity was abolished by incubation with normal mouse or rabbit IgG as controls (data not shown). Bar: 100 μm.

in the mouse oviduct (Eroschenko 1982). The teratogenic and carcinogenic effects of perinatal exposure to diethylstilbestrol (DES), a synthetic estrogen, on the mouse and rat oviduct are well documented (Herbst & Bern 1981, Newbold *et al.* 1983, Iguchi 1992), and ERα KO mice showed resistance to those adverse effects of DES (Couse *et al.* 2001). Therefore, the increase in oviductal

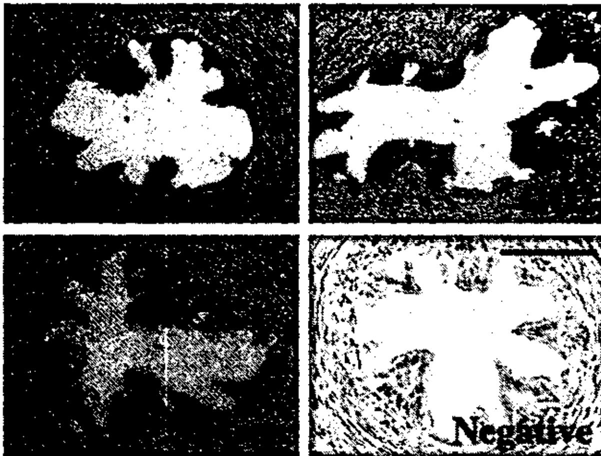

Figure 6 Change in PR-A+B immunoexpression in the IST during the estrous cycle and early pregnancy. Immunohistochemistry for PR-A+B exhibited marked staining in epithelial, stromal and muscle cells in the IST at diestrous stage (Diestrus). However, they decreased in intensity to moderate at estrus stage (Estrus). In pregnant rats, PR-A+B staining was observed at moderate levels in epithelial, stromal and muscle cells in the GD 0 oviduct, similar to the estrus oviduct (data not shown), and increased to marked levels on GD 2. PR-A+B immunoreactivity was abolished by incubation with normal mouse IgG as negative control (Negative). Bar: 100 μm.

ERα may have a critical role in the neonatal proliferation and cytodifferentiation of oviductal cells in response to endogenous and exogenous estrogens. If estrogen regulates epithelial ciliary formation directly via epithelial ERα in the neonatal oviduct, this potential mechanism may be different from that in ERα-negative epithelial cells of the adult oviduct discussed below. In contrast, although the physiological role of PRs and AR has not been clearly demonstrated in the development of the rat oviduct, both epithelial and stromal cells could be targets for endogenous progestins and androgens, and exogenous PRs and AR modulators. Especially, because epithelial cells of neonatal IST markedly expressed PRs, they could be a critical target for progestin action.

Oviductal cilia are believed to have the critical role in ovum transport through oviduct to uterus in cycling and pregnant rats (Halbert *et al.* 1989). Estrogen accelerated ovum transport in pregnant rats, and concomitant P4 treatment blocked the estrogen-induced events (Banik & Pincus 1964, Fuentealba *et al.* 1988). In the cycling and pregnant rats, ovariectomy or hypophysectomy,

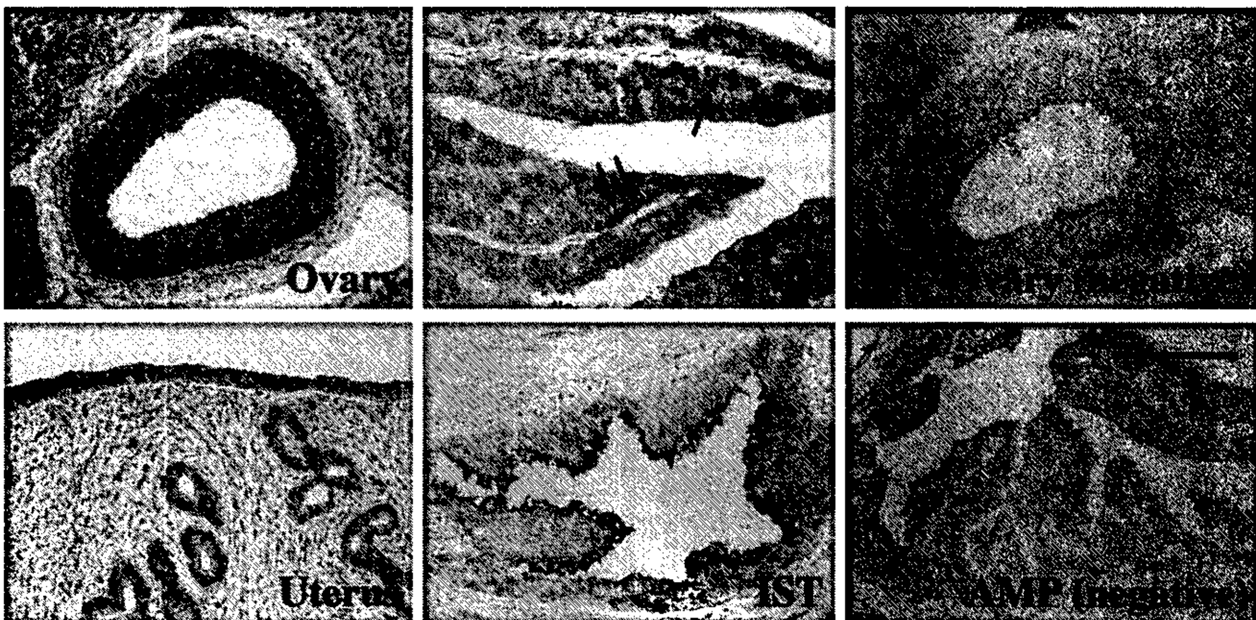

Figure 7 Immunohistochemical localization of ERβ in the ovary (Ovary), uterus and oviduct. Marked staining was found in ovarian granulosa cells. In the uterus, ERβ staining was detected at moderate and slight levels in luminal and glandular epithelia, and peripheral stroma respectively (Uterus). Also, few epithelial cells in the AMP showed positive staining for ERβ (arrows), but no positive cell was noted in the IST. ERβ immunoreactivity was abolished by incubation with normal rabbit immunoglobulin fraction in the ovary and AMP (Negative). Bar: 200 μm for the ovary, 100 μm for the uterus, AMP and IST.

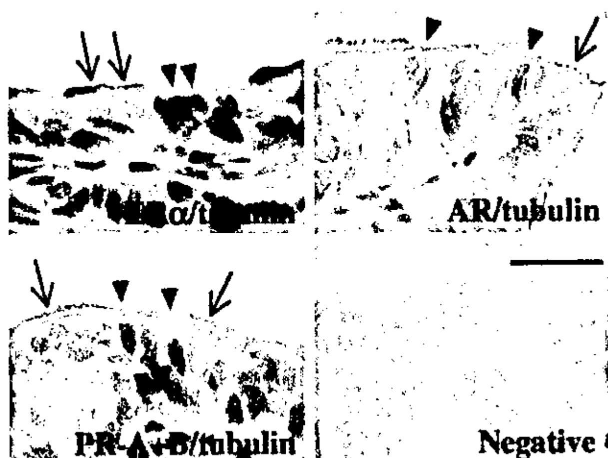

Figure 8 Double immunohistochemical localization of ERα, PR-A+B or AR, with β-tubulin IV in the AMP of the diestrous oviduct. Staining of ERα, PR-A+B or AR (brown), and β-tubulin IV (red) were not found to be co-localized in the same surface epithelial cells. Arrowheads indicate ERα-, PR-A+B- or AR-positive, but β-tubulin IV-negative, cells. Arrows indicate ERα-, PR-A+B- or AR-negative, but β-tubulin IV-positive, cells. Immunoreactivity was abolished by incubation with normal mouse and rabbit IgG (Negative). Bar: 20 μm.

and treatment with the aromatase inhibitor 4-hydroxyandrostenedione, caused delayed ovum transport due to reduction of estrogen production (Wu *et al.* 1971, Forcelledo & Croxatto 1986, 1988). In contrast, treatment with the PR antagonist RU486 caused accelerated ovum transport in rats (Fuentealba *et al.* 1987). Thus, estrogen and P4 may have roles in ovum transport by regulating oviductal ciliogenesis in rats. In the present study, however, a lack of ERα and PRs in ciliated epithelial cells was shown by double immunostaining with β-tubulin IV, suggesting no possibility of direct regulation of ciliogenesis of ciliated cells by estrogen and P4. We hypothesized, therefore, two possible mechanisms of ciliogenesis regulation by estrogen and P4 in the rat oviduct: (i) interaction between epithelial and stromal cells via intermediate molecules which are produced by ERα- and PRs-positive stromal cells, and (ii) cytodifferentiation from ERα- and PRs-positive nonciliated (progenitor?) epithelial cells to ciliated epithelial cells with ciliation and subsequent loss of ERα and PRs. Since a fundamental role of epithelial–stromal tissue/cell interaction was clearly demonstrated in estrogen action on cell proliferation and epithelial PR regulation in mouse uterus and vagina (Cooke *et al.* 1997, 1998, Buchanan *et al.* 1998, Kurita *et al.* 2000*a,b*, 2001), there is likely to be a similar situation in the oviduct. In contrast, change of cell phenotype from secretory to ciliated cells with estrogen treatment was demonstrated in primary culture of human oviductal epithelial cells (Comer *et al.* 1998). Moreover, Reeder & Shirley (1999) suggested the possibility of transformation from ciliated cells to secretory cells following loss of cilia in an electron microscopic study. Further elucidation of this matter is necessary.

Although effects of androgens on the oviduct still remain obscure, 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 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 was not 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, if AR and ERα co-localize in the identical cell.

In conclusion, the cell- and region-dependent ERα, ERβ, PRs and AR expressions were immunohistochemically determined in the rat oviduct throughout various physiological conditions. This study could be helpful for understanding the molecular and cellular mechanisms underlying estrogen, progestin and androgen actions on the rat oviduct.

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Estrogen Receptors (α and β) and 17β-Hydroxysteroid Dehydrogenase Type 1 and 2 in Thyroid Disorders: Possible *In Situ* Estrogen Synthesis and Actions

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Both epidemiological and experimental findings suggest the possible roles of sex steroids in the pathogenesis and/or development of various human thyroid disorders. In this study, we evaluated the expression of estrogen receptors (ER) α and β in normal thyroid glands (N = 25; female: n = 13, male: n = 10, unknown: n = 2) ranging in age from fetus to adult. Furthermore, using immunohistochemistry, we investigated the expression of ERα and β in 206 cases of thyroid disorders, including 24 adenomatous goiters, 23 follicular adenomas, and 159 thyroid carcinomas. In addition, we also studied the mRNA expression of ERα and β and 17β-hydroxysteroid dehydrogenase Type 1 and 2, enzymes involved in the interconversion between estrone and estradiol, using reverse transcription polymerase chain reaction (RT-PCR), in 48 of these 206 cases (10 adenomatous goiters, 10 follicular adenomas, and 28 papillary thyroid carcinomas) in which fresh frozen tissues were available for examination to further elucidate the possible involvement of intracrine estrogen metabolism and/or actions in thyroid disorders. ERα labeling index, or percentage of cells immunopositive for ERα, was significantly higher in adenomatous goiter (14.2 ± 6.4), follicular adenoma (13.4 ± 5.1), and thyroid carcinoma (16.4 ± 2.1) than in normal thyroid gland (0; P < .05). Few follicular cells were positive for ERα in normal thyroid glands. In papillary carcinoma, ERα labeling index was significantly higher in premenopausal women (28.1 ± 4.5) than in postmenopausal women (14.2 ± 2.9) and in men of various ages (7.6 ± 2.7; P < .05). In other histological types of thyroid carcinoma, no significant correlations were detected. ERβ immunoreactivity was detected in both follicular and C-cells of normal thyroid glands, including those in developing fetal thyroid glands. In addition, ERβ immunoreactivity was detected in the nuclei of various thyroid lesions. But no significant correlations were detected between ERβ labeling index and clinicopathological findings including age, menopausal status, gender, and/or histological type of thyroid lesions. 17β-hydroxysteroid dehydrogenase Type 1 expression was detected in 31/48 (64.0%) of the cases examined, whereas Type 2 was detected only in 3/46 (6.3%) of all the cases examined. These results demonstrated that estrogens may influence the development, physiology, and pathology of human thyroid glands, and these effects, especially through ERα, may become more pronounced in neoplasms, particularly in papillary carcinoma arising in premenopausal women.

KEY WORDS: Estrogen receptors, Human thyroid lesions, 17β-hydroxysteroid dehydrogenase, Immunohistochemistry, Labeling index, Reverse transcription polymerase chain reaction.

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Biological effects of estrogens are generally mediated through an initial interaction with the estrogen receptor (ER), a member of the superfamily of nuclear receptors. Identification of ER is an initial step in understanding the estrogenic effects on various

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tumors. ER has been identified, for instance, in a wide range of human neoplasms, including carcinoma arising in colon (1), lung (2), pancreas (3), and other organs classically considered not to be targets for estrogens. However, their biological significance in these tumors has not been fully elucidated. Thyroid tumors are well known to occur with approximately three times more frequency in women than in men (4). Given the higher incidence and relatively better prognosis of thyroid carcinomas diagnosed in women (5), especially in premenopausal women, it is reasonable to speculate that these thyroid lesions may be influenced by sex hormones, especially estrogens, in their pathogenesis and/or development.

In addition to the classical ERα isoform, a second isoform of ER or ERβ, has been recently identified in humans (6). ERβ has been demonstrated to be widely distributed in various human tissues (7, 8), including many organs of the human fetus (9, 10) in contrast to ERα. Several investigators have demonstrated the presence of ERα in thyroid tissues from human thyroid tumors and normal thyroid glands (11–13), but not that of ERβ. In addition, Valle *et al.* (11) reported an overexpression of aromatase, the enzyme involved in conversion of androgens to estrogens, in several human thyroid lesions. However, results of these studies on ERα in normal and pathologic human thyroids have been inconsistent and could not necessarily account for the marked prevalence of thyroid lesions among female subjects. 17β-Hydroxysteroid dehydrogenase catalyzes the reversible interconversion between estrone and estradiol. However, 17β reduction and oxidation of estrogens is catalyzed by different 17β-hydroxysteroid dehydrogenase isozymes. 17β-hydroxysteroid dehydrogenase Type 1 mainly catalyzes the conversion of inactive estrogen, estrone, to the biologically active estrogen, estradiol (14–16), whereas 17β-hydroxysteroid dehydrogenase Type 2 predominantly catalyzes the conversion of estradiol to estrone (17). 17β-hydroxysteroid dehydrogenase Type 1 and 2 both are known to regulate the *in situ* levels of estradiol, and subsequently to modulate estrogenic actions in estrogen target tissues. Examination of 17β-hydroxysteroid dehydrogenase Type 1 and 2 in human thyroid lesions, in addition to aromatase and ER isoforms, therefore, become very important in obtaining a better understanding of the local or intracrine regulation of estrogenic actions in human thyroid lesions. Therefore, in this study, we first examined the expression of ERα and β in normal thyroid glands obtained from fetal, pediatric, and adult patients. We then studied the expression of ERα and β, and 17β-hydroxysteroid dehydrogenase Types 1 and 2 in adenomatous goiters (n = 24), follicular adenomas (n = 23), and thyroid carcinomas (n = 159), using immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR) to elucidate the possible roles of estrogens in the development, physiology, and pathology of the human thyroid gland.

MATERIALS AND METHODS

Tissue Preparation

Human fetal tissues (10–21 weeks gestational age) were obtained after elective termination in normal pregnant women at Tohoku University Hospital and Nagaike Maternal Clinic (Sendai, Japan). Informed consent was obtained from these pregnant women before elective termination. The ages of the fetuses were estimated by the last menstrual date, body weight, and/or crown-rump length. Human fetal tissues of 24, 27, 37, and 39 weeks of age were obtained at the time of autopsy at Tohoku University Hospital. The ethics committee of Tohoku University School of Medicine approved this research protocol. The time elapsed from demise to the removal of the tissues ranged from 30 minutes to 1 hour in the case of elective termination, and from 2 to 6 hours in autopsy cases. After careful evaluation, these specimens did not appear to have any significant histopathological abnormalities. The specimens for immunohistochemistry were fixed in 10% neutral formalin for 18 hours at 4° C and then embedded in paraffin.

Patients and Tissues

Paraffin-embedded blocks from 206 patients who underwent thyroidectomy from 1990 to 2000 at Tohoku University Hospital, Sendai City Hospital, and Itou Hospital (Tokyo, Japan) were used for immunohistochemical studies. The mean age of the patients was 52.8 ± 17.5 years old (range, 12–86 y). Twenty-four cases of adenomatous goiter (18 females, 6 males; 53.5 ± 1.0 y), 23 cases of follicular adenoma (17 females, 6 males; 48.1 ± 14.9 y), and 159 cases of carcinoma (116 females, 43 males; 53.8 ± 17.5 y) were retrieved from the surgical pathology files at all three hospitals mentioned above. Carcinoma cases that we examined included 100 papillary (79 females, 21 males; 53.5 ± 17.6 y), 14 follicular (8 females, 6 males; 47.6 ± 17.5 y), 25 anaplastic (15 females, 10 males; 65.2 ± 9.1 y), and 20 medullary (14 females, 6 males; 45.7 ± 18.6 y) carcinomas (Table 1). All patients examined in this study received neither irradiation nor chemotherapy before surgery. The World Health Organization classification of thyroid lesions and the general rules for the description of thyroid cancer in Japan (18) were used as the basis for histological classification of thyroid tumors investigated in this study.

TABLE 1. Histological Diagnosis of Paraffin-Embedded Thyroid Tissues

	Women		Men	Total
	Premenopausal	Postmenopausal		
Normal	3	3	5	11
Adenomatous goiter	7	11	6	24
Follicular adenoma	10	7	6	23
Carcinoma	47	69	43	159
Papillary	35	44	21	100
Follicular	5	3	6	14
Anaplastic	0	15	10	25
Medullary	7	7	6	20
Total	67	91	59	217

Fresh thyroid tissues were available for total RNA extraction and RT-PCR examination in 48 patients with thyroid disease from 1997 to 2000 at the three institutions above. Thyroid tissues were immediately frozen in liquid nitrogen and stored at −80° C until use for the RT-PCR study. The mean age of the patients was 53.3 ± 15.5 years (range, 20–86 y). These 48 cases included 28 papillary carcinomas (21 females, 7 males; 55.8 ± 16.2 y), 10 follicular adenomas (8 females, 2 males; 47.2 ± 14.6 y), and 10 adenomatous goiters (9 females, 1 males; 52.7 ± 13.9 y). All of these cases were histologically confirmed.

Antibodies

The polyclonal antibody for ERβ was raised in rabbit against synthesized peptides of the C-terminal region of ERβ (CSPAEDSKSKEGSQN-PQSQ). This antibody was purified on affinity columns bound with the synthetic peptide. The characterization of this antibody was confirmed by Western blotting (19), and use of the ERβ antibody for immunohistochemistry has been previously reported. The monoclonal antibody for ERα (ER1D5) was purchased from Immunotech (Marseille, France).

Immunohistochemistry

In this study, immunohistochemical analyses were performed using the streptavidin-biotin amplification method and a Histofine Kit (Nichirei, Tokyo, Japan) for ERα, and Envision+ (DAKO, Carpinteria, CA) for ERβ. For antigen retrieval, slides were deparaffinized and heated in an autoclave at 120° C for 5 minutes in a citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0) for the immunostaining of ERα and ERβ. The dilutions of primary antibodies used in this study were as follows: ERα 1/100, and ERβ 1/100. The antigen–antibody complex was visualized with 3.3-diaminobenzidine (3,3'-diaminobenzidine) solution M 3,3'-diaminobenzidine, 50 mM Tris-HCl buffer [pH 7.6], and 0.006% H_2O_2, and counterstained with hematoxylin. Breast cancer was used as a positive control for ERα, and normal breast was also used as a positive control for ERβ. Normal rabbit or mouse IgG was used instead of the primary antibodies as a negative control. No specific immunoreactivity was detected in these sections.

RT-PCR Analysis

Total RNA was extracted by homogenizing tissue specimens in guanidium thiocyanate, followed by ultracentrifugation in cesium chloride. Total RNA was spectrophotometrically quantified at 260 nm. An RT-PCR kit (SUPERSCRIPT Preamplification system, Gibco-BRL, Grand Island, NY) was employed in the synthesis and amplification of cDNA. cDNAs were synthesized from 5 μg of total RNA using random hexamer primer. Reverse transcription was carried out for 60 minutes at 42° C with SUPERSCRIPT II reverse transcriptase. After an initial 1-minute denaturation step at 95° C, 40 cycles of PCR were carried out on a Light Cycler PCR machine (Roche Diagnostics GmbH, Mannheim, Germany) under the following condition: 0 seconds of denaturation at 95° C, 15 seconds of annealing at 62° C for ERα or at 60° C for ERβ and GAPDH, followed by a 15-second extension at 72° C. The primer sequences used in this study are listed in Table 2 (21–24). After PCR, the products were resolved on a 2% agarose ethidium bromide gel, and images were captured with Polaroid film under ultraviolet transillumination. In initial experiments, amplified PCR products were purified and subjected to direct sequencing (ABI PRISM Biosystems, Foster City, CA; and ABI Prism 310 Genetic Analyzer) to verify amplification of the correct sequences. As a positive control, T-47D breast cancer cells were used for ERα and ERβ; frozen placental tissue was used as a positive control for 17β-HSD Type 1 and 2. In the present study, we tested for the presence of exogenous and/or non-gene-specific contaminant DNA by synthesizing cDNA in the absence of reverse transcriptase and also by performing a standard PCR without cDNA substrate. We observed no amplification of gene-specific products under either of these conditions.

Scoring of Immunoreactivity

Scoring of ERα and ERβ in tumor cells was performed on high-power field (400×) using a standard light microscope. In each case, the fields for examination were simultaneously determined by two of the authors (WK and TS) using a double-headed light microscope. More than 1000 tumor cells were counted independently by the two authors above. Nuclear immunoreactivity was considered positive regardless of immunointensity. The

TABLE 2. Primer Sequences Used in RT-PCR Analysis

cDNA	Sequence	Position in cDNA	Size (bp)	Reference Number
ERα	Forward 5'AAGAGCTGCCAGGCCTGCC	702–720	167	21
	Reverse 5'TTGGCAGCTCTCATGTCTCC	850–869		
ERβ	Forward 5' GCATGGAACATCTGCTCAAC	1513–1532	229	22
	Reverse 5' ACGCTTCAGCTTGTGACCTC	1721–1740		
17β-HSD type 1	Forward 5'CTCTGGCGCTGCCCCAACAC	598–615	352	23
	Reverse 5'GGACCTGCTGGTGTGTAAC	264–282		
17β-HSD type 2	Forward 5'CTTTGTGACCTCCACAGTTC	667–686	418	23
	Reverse 5'GGTGTCATGCTTCCTCATGT	269–288		
GAPDH	Forward 5'TGAACGGGAAGCTCACTGG	731–750	307	24
	Reverse 5' TCCACCACCCTGTTGCTGTA	1018–1038		

percentage of positive immunoreactivity, that is, labeling index, was determined. When interobserver differences were >5%, the cases were simultaneously re-evaluated using a double-headed light microscope. When interobserver differences were <5%, the mean values of these two observed data were determined as the labeling index for that case. Interobserver differences in our study were <1%.

Statistical Analysis

The gender and menopausal status of the patients in this study were tentatively classified into three groups (premenopausal women, postmenopausal women, and men of various ages). Values for each category and the labeling index for ERα and ERβ are presented as mean ± standard error of means (SEM). Associations between the labeling index for ERα and ERβ and each histological category were evaluated using an ANOVA test. A P-value of <.05 was considered significant.

RESULTS

Immunohistochemistry

Results are summarized in Tables 3 and 4.

ERα

ERα immunoreactivity was detected in the nuclei of invasive ductal carcinoma employed as positive control of immunostain. Very few follicular cells were positive for ERα in the normal thyroid gland of both women and men. ERα immunoreactivity was not detected in the nuclei of follicular cells in developing fetal thyroid glands. However, ERα immunoreactivity was detected in the nuclei of parenchymal cells in thyroid disorders (Fig. 1, A–D). The numbers of ERα-positive cases were as follows: 9/24 (37.5%: 6/18 [33.3%] females, 3/6 [50%] males) in adenomatous goiters, 6/23 (26.1%: 4/17 [23.5%] females, 2/6 [33.3%] males) in follicular adenomas, and 74/159 (46.5%: 58/116 [50.0%] females, 16/43 [37.2%] males) in carcinomas. ERα labeling index or the percentage of ERα-positive cells was as follows: 14.2 ± 6.4 in adenomatous goiter, 13.4 ± 5.1 in follicular adenoma, 17.8 ± 2.7 in papillary carcinoma, 13.3 ± 6.3 in follicular carcinoma, 18.8 ± 5.7 in anaplastic carcinoma, and 5.5 ± 3.8 in medullary carcinoma.

Results of the correlation between ERα labeling index and clinicopathological parameters are summarized in Table 3. ERα labeling index was significantly higher in adenomatous goiter (14.2 ± 6.4), follicular adenoma (13.4 ± 5.1), and thyroid carcinoma (16.4 ± 2.1%) than in normal thyroid gland (0; $P < .05$). In papillary carcinoma, ERα labeling index was significantly higher in premenopausal women (28.1 ± 4.5) than in postmenopausal women (14.2 ± 2.9) and men of various ages (7.6 ± 2.7; $P < .05$). With respect to anaplastic carcinoma, ERα labeling index tends to be higher in men of various ages (34.7 ± 10.1) than in postmenopausal women (8.2 ± 5.4), but the differences did not reach statistical significance. No significant correlations were detected in other histological types. Clinical

TABLE 3. Mean ± SEM of Labeling Indexes of ERα immunoreactivity

	Women		Men	Total
	Premenopausal	Postmenopausal		
Normal	0	0	0	0
Adenomatous goiter	8.2 ± 6.9	10.5 ± 6.3	23.2 ± 13.0	14.2 ± 6.4
Follicular adenoma	6.8 ± 4.8	23.5 ± 4.1	11.8 ± 10.8	13.4 ± 5.1
Carcinoma				
Papillary	28.1 ± 4.5	14.2 ± 2.9	7.6 ± 2.7	17.8 ± 2.7
Follicular	20.3 ± 10.5	14.3 ± 14.3	21.8 ± 10.4	13.3 ± 6.3
Anaplastic	—	8.2 ± 5.4	34.7 ± 10.1	18.8 ± 5.7
Medullary	6.3 ± 5.5	10.2 ± 9.4	0	5.5 ± 3.8

TABLE 4. Mean ± SEM of LIs of ERβ immunoreactivity

	Women		Men	Total
	Premenopausal	Postmenopausal		
Normal	12.5 ± 2.6	13.0 ± 3.9	21.6 ± 3.0	22.4 ± 2.5
Adenomatous goiter	17.2 ± 14.5	12.2 ± 7.4	16.2 ± 9.2	15.2 ± 7.3
Follicular adenoma	29.0 ± 5.2	23.5 ± 10.8	34.0 ± 19.0	29.2 ± 8.2
Carcinoma				
Papillary	15.4 ± 4.5	17.2 ± 3.2	19.6 ± 6.2	17.0 ± 2.9
Follicular	12.5 ± 11.2	23.3 ± 13.6	13.6 ± 6.2	15.5 ± 5.3
Anaplastic	—	38.4 ± 7.1	42.0 ± 9.5	39.9 ± 5.6
Medullary	7.2 ± 2.7	40.5 ± 16.2	24.3 ± 13.7	24.0 ± 7.9

follow-up data of these patients with thyroid carcinoma also were not available for present study.

ERβ

ERβ nuclear immunoreactivity was detected widely in both follicular or parenchymal and C-cells of normal thyroid glands, including those in the developing fetal thyroid glands (Fig. 1, E–F). In addition, ERβ immunoreactivity was detected in the nuclei of various thyroid lesions. The number of ERβ-positive cases or percentages of ERβ-positive cases were as follows: 9/24 (37.5%: 6/18 [33.3%] females, 3/6 [50%] males) in adenomatous goiters, 6/23 (26.1%: 4/17 [23.5%] females, 2/6 [33.3%] males) in follicular adenomas and 74/159 (46.5%: 70/116 [60.3%] females, 25/43 [58.1%] males) in carcinomas. ERβ labeling index was as follows: 17.7 ± 4.6 in normal thyroid gland, 15.2 ± 7.3 in adenomatous goiter, 29.2 ± 8.2 in follicular adenoma, 17.0 ± 2.9 in papillary carcinoma, 15.5 ± 5.3 in follicular carcinoma, 39.9 ± 5.6 in anaplastic carcinoma, and 24.0 ± 7.9 in medullary carcinoma.

Results of the correlation between ERβ labeling index and clinicopathological parameters of patients were summarized in Table 4. ERβ labeling index tended to be higher in anaplastic carcinoma (39.9 ± 5.6) than in other histological types, but the differences did not reach statistical significance. There were no significant correlations between ERβ labeling index and patient age, menopausal status, gender, benign and malignant tumors, histological type and follow-up data (data not shown).

RT-PCR

Messenger RNA (mRNA) expression for ERα, ERβ, and 17β-hydroxysteroid dehydrogenase Type 1 and 2 was detected as a signal gene-specific band (168 bp for ERα, 228 bp for ERβ, 352 bp for 17β-hydroxysteroid dehydrogenase Type 1, and 418 bp for 17β-hydroxysteroid dehydrogenase Type 2; Fig. 2) in 48/48 (100%), 48/48 (100%), 31/48 (64.6%), and 3/48 (6.3%) specimens examined, respectively. Results of RT-PCR analysis were consistent with those of immunohistochemistry in each case.

No significant correlations were detected between 17β-hydroxysteroid dehydrogenase Type 1 and the labeling index for ERα and β, or between 17β-hydroxysteroid dehydrogenase Type 2 and the labeling index for ERα and β.

DISCUSSION

In our present study, ERα immunoreactivity was not detected in the normal thyroid gland, but ERβ immunoreactivity was detected in the nuclei of follicular epithelial cells and C-cells throughout the development of the normal thyroid gland. The presence of ERβ previously has been demonstrated in the normal rat thyroid gland by immunohistochemistry (24). In addition, ERβ has very recently been demonstrated in adult normal human thyroid gland by immunohistochemistry (8). Results from our present study are also consistent with those from previous studies. In our study, ERβ immunoreactivity was detected throughout the development of the human thyroid gland from 11 gestational weeks. In normal pregnancy, large quantities of estrogens are produced by placental syncytiotrophoblasts, which subsequently move into both the fetal and maternal circulation. The effects of estrogens on thyroid development have not been well studied, but results of our present study suggest that estrogens may also be involved in the development of fetal thyroid glands. These effects are most likely to be predominantly mediated via ERβ.

ERα immunoreactivity was detected in the nuclei of thyroid parenchymal cells of various thyroid lesions. The presence of ERα has previously been examined in human thyroid neoplasms by a number of investigators (11–13, 25–27). However, results from these studies were inconsistent, possibly because of the different methods employed. In our present study, we employed immunohistochemistry in combination with labeling index, one of the most reliable methods for detecting ERα in clinical specimens, and also RT-PCR. ERα labeling index was significantly higher in adenomatous goiters, follicular adenomas and thyroid carcinomas than in normal thyroid glands. In the papillary carcinoma,

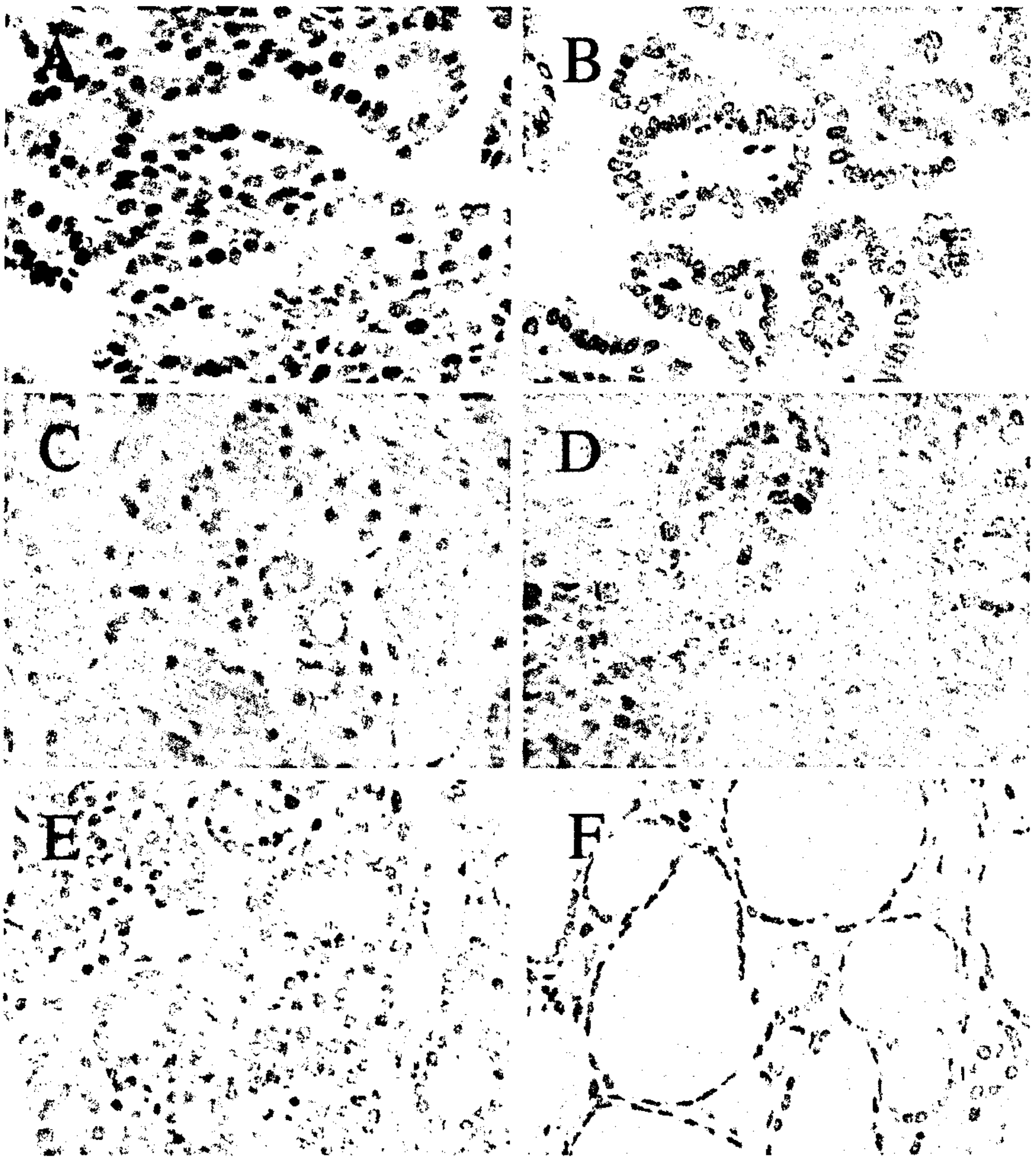

FIGURE 1. Immunohistochemistry for ERα in (A, B) papillary carcinoma, (C) adenomatous goiter obtained from premenopausal women, respectively, and (D) anaplastic carcinoma obtained from postmenopausal women. Immunohistochemistry for ERβ in (E) the fetal thyroid gland (14 gestational weeks) and (F) in the adult thyroid gland.

ERα labeling index was significantly higher in post-puberty/premenopausal women than in postmenopausal women and men of various ages. A statistically significant association between ERα and the age of the patients was not detected in other types of thyroid lesions. Estradiol has been demonstrated to stimulate cell proliferation of FRTL-5 rat cells, a well-established cell line derived from thyroid papillary carcinoma (28), and the human thyroid papillary carcinoma cell line, HTC-TSHr (29). These results all suggest that higher estrogen concentrations in the serum may be responsible for the relatively high incidence of thyroid papillary carcinoma in premenopausal women. In addition, a higher level of ERα labeling index may also be involved in the activation of

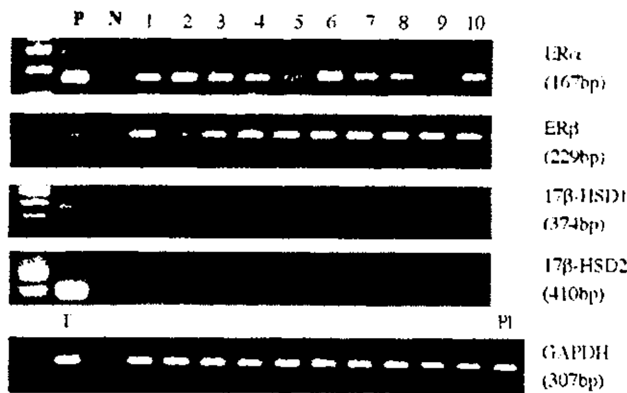

FIGURE 2. RT-PCR analysis for ERα, ERβ, and 17β-HSD Type 1 and Type 2 in human thyroid tumors. mRNA expression for ERα, ERβ, 17β-HSD Type 1, Type 2, and GAPDH were detected as a single gene-specific band (168 bp for ERα, 228 bp for ERβ, 352 bp for 17β-HSD Type 1, 418 bp for 17β-HSD Type 2 and 307 bp for GAPDH). P, Positive controls (T-47D for ERα and ERβ, placenta for 17β-HSD Type 1 and Type 2); T, T-47D; Pl, placenta; N, negative control (no cDNA substrate). All thyroid lesion specimens were obtained from (pre-) or (post-) menopausal women with the exception of 6 and 10, which were obtained from male patients. 1–2, adenomatous goiter (post-); 3–4, papillary carcinoma (pre-); 5, adenomatous goiter (pre-); 6, papillary carcinoma; 7, follicular adenoma (pre-); 8, adenomatous goiter (pre-); 9, papillary carcinoma (post-); 10, papillary carcinoma.

tumorigenesis of papillary carcinoma in premenopausal women, but it awaits further investigations for clarification.

The status of ERβ has not been well studied in human thyroid disorders. In our present study, ERβ nuclear immunoreactivity was detected relatively widely in thyroid neoplastic parenchymal cells. There were, however, no significant correlations between ERβ immunoreactivity and any clinicopathological parameters examined, including patient age, menopausal status, gender, histological type, and fetal developmental stage. In addition, ERβ is widely distributed in follicular cells and C-cells of the normal thyroid gland throughout development. These results above suggest that estrogenic effects via ERβ play important roles in the maintenance of homeostasis of thyroid follicles, and possibly of thyroid hormone biosynthesis.

P450 aromatase converts androstenedione to estrone and testosterone to estradiol. Recent studies demonstrated that bioactive estrogen, estradiol, is produced locally in breast carcinoma (30). Estrogen also has been reported to be produced locally in human thyroid tumors (11). In addition, aromatase immunoreactivity has been demonstrated to be expressed in the cytoplasm of follicular epithelial cells (11). In our present study, the expression of 17β-hydroxysteroid dehydrogenase Types 1 and 2 was demonstrated by RT-PCR. Especially, 17β-hydroxysteroid dehydrogenase Type 1 was detected in 31/48 (64.0%) of the cases examined. Expression of ER, aromatase, and 17β-hydroxysteroid dehydrogenase Type 1 has been demonstrated in human thyroid lesions as in human breast carcinoma (30). This mechanism can effectively produce estrogens *in situ* from circulating androgens and exert their effects locally. Further investigations are required to fully elucidate the roles and mechanisms of estrogenic actions with respect to pathogenesis and/or development of human thyroid disorder.

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Vitamin K_2 Regulation of Bone Homeostasis Is Mediated by the Steroid and Xenobiotic Receptor SXR*

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Vitamin K_2 is a critical nutrient required for blood clotting that also plays an important role in bone formation. Vitamin K_2 supplementation up-regulates the expression of bone markers, increases bone density *in vivo*, and is used clinically in the management of osteoporosis. The mechanism of vitamin K_2 action in bone formation was thought to involve its normal role as an essential cofactor for γ-carboxylation of bone matrix proteins. However, there is evidence that suggests vitamin K_2 also has a transcriptional regulatory function. Vitamin K_2 bound to and activated the orphan nuclear receptor SXR and induced expression of the SXR target gene, *CYP3A4*, identifying it as a *bona fide* SXR ligand. Vitamin K_2 treatment of osteosarcoma cells increased mRNA levels for the osteoblast markers bone alkaline phosphatase, osteoprotegerin, osteopontin, and matrix Gla protein. The known SXR activators rifampicin and hyperforin induced this panel of bone markers to an extent similar to vitamin K_2. Vitamin K_2 was able to induce bone markers in primary osteocytes isolated from wild-type murine calvaria but not in cells isolated from mice deficient in the SXR ortholog *PXR*. We infer that vitamin K_2 is a transcriptional regulator of bone-specific genes that acts through SXR to favor the expression of osteoblastic markers. Thus, SXR has a novel role as a mediator of bone homeostasis in addition to its role as a xenobiotic sensor. An important implication of this work is that a subset of SXR activators may function as effective therapeutic agents for the management of osteoporosis.

Osteoporosis is a common disease affecting the elderly, particularly postmenopausal women, although a significant minority of older men is also affected. It is defined as the gradual reduction in bone strength with advancing age that is manifested by such observations as bone fracture following minimal trauma (1–3). Several types of agents are used clinically in the United States to prevent or treat osteoporosis. These include estrogen/progestin replacement therapy, calcitonin, bisphosphonate, and selective estrogen receptor modulators (4). Vitamin K_2 was first reported to promote fracture healing in 1960 (5), and several studies showed that vitamin K_2 is closely associated with increased bone formation (6, 7) and decreased bone resorption (8–13). Low levels of dietary vitamin K are associated with increased risk of fractures (14–16). Accordingly vitamin K_2 is used clinically in Japan to treat osteoporosis either alone or in conjunction with 1α,25-$(OH)_2$ vitamin D_3 (17–20).

Vitamin K_2 is a family of naphthoquinones, the most biologically important of which is menatetrenone (for a review, see Ref. 21). Vitamin K_2 was discovered as a critical nutrient required for blood clotting. It acts as a cofactor for the microsomal γ-carboxylase that facilitates the post-translational conversion of glutamic acid to γ-carboxyglutamyl (Gla) residues (for a review, see Ref. 14). Post-translational conversion of 9–12 Glu to Gla residues is required for the function of proteins such as prothrombin and Factors VII, IX, and X in the blood clotting cascade (for reviews, see Refs. 21 and 22). In addition, Gla-containing proteins such as osteocalcin and matrix Gla protein are abundant in bone tissues where they are thought to play important roles in regulating mineralization (for reviews, see Refs. 23 and 24).

Recent studies have demonstrated that the orphan nuclear receptor SXR[1] (25) (also known as PXR (26), PAR (27), and NR1I2) plays a central role in the transcriptional regulation of xenobiotic detoxifying enzymes and transporters such as *CYP3A4* and *MDR1* (28–32). SXR is activated by a diverse array of pharmaceutical agents including Taxol, rifampicin, SR12813, clotrimazole, phenobarbital, hyperforin (33–35), the herbal antidepressant St. John's wort (36), and peptide mimetic human immunodeficiency virus protease inhibitors such as ritonavir (28). These studies indicate that SXR functions as a xenobiotic sensor to coordinately regulate drug clearance in the liver and intestine. Indeed gene knockout studies have confirmed a role for SXR in regulating the metabolism of en-

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[1] The abbreviations used are: SXR, steroid and xenobiotic receptor; PXR, pregnane X receptor; CYP, cytochrome P-450; RT-PCR, reverse transcriptase PCR; QRT-PCR, quantitative real time RT-PCR; RIF, rifampicin; ALP, alkaline phosphatase; OPN, osteopontin; MGP, matrix Gla protein; OPG, osteoprotegerin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; SRC-1, steroid receptor coactivator-1; ACTR, activator for thyroid hormone and retinoid receptors; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; F, forward; R, reverse; PCN, pregnenolone 16α-carbonitrile; PBP, peroxisome proliferator-activated receptor (PPAR)-binding protein; GRIP, glucocorticoid receptor-interacting protein; SMRT, silencing mediator of retinoid and thyroid receptors; NCoR, nuclear receptor corepressor; CHX, cycloheximide; WT, wild-type.