Immunolocalisation of oestrogen receptor beta in human tissues

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ABSTRACT

Oestrogens exert their actions via specific nuclear protein receptors that are members of the steroid/thyroid receptor superfamily of transcription factors. Recently, a second oestrogen receptor (ERβ) has been cloned, and using reverse transcription-PCR and immunohistochemistry it has been shown to have a wide tissue distribution in the rat that is distinct from the classical oestrogen receptor, ERα. Using commercial polyclonal antisera against peptides specific to human ERβ, we have determined the sites of ERβ expression in archival and formalin-fixed human tissue and compared its expression with that of ERα. ERβ was localised to the cell nuclei of a wide range of normal adult human tissues including ovary, Fallopian tube, uterus, lung, kidney, brain, heart, prostate and testis. In the ovary, ERβ was present in multiple cell types including granulosa cells in small, medium and large follicles, theca and corpora lutea, whereas ERα was weakly expressed in the nuclei of granulosa cells, but not in the theca nor in the copora lutea. In the endometrium, both ERα and ERβ were observed in luminal epithelial cells and in the nuclei of stromal cells but, significantly, ERβ was weak or absent from endometrial glandular epithelia. Epithelial cells in most male tissues including the prostate, the urothelium and muscle layers of the bladder, and Sertoli cells in the testis, were also immunopositive for ERβ. Significant ERβ immunoreactivity was detected in most areas of the brain, with the exception of the hippocampus – a tissue that stained positively for ERα. In conclusion, the almost ubiquitous immunohistochemical localisation of ERβ indicates that ERβ may play a major role in the mediation of oestrogen action. The differential expression of ERα and ERβ in some of these tissues suggests a more complex control mechanism in oestrogenic potential than originally envisioned.

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INTRODUCTION

Steroid action is mediated by specific intracellular receptors which bind ligand and transclocate to the nucleus to activate gene transcription (Brinkmann 1994). The recently discovered beta isoform of oestrogen receptor (ERβ) that shows ligand specificity for oestrogens is expressed in a number of rat tissues (Saunders *et al*. 1997). However, some of the data originally reported for the tissue distribution of ERβ in rat and mouse using reverse transcription (RT)-PCR and RNase protection assay techniques (Couse *et al*. 1997*a*, Kuiper *et al*. 1997, Mitchner *et al*. 1997) differ from the data obtained using ERβ immunohistochemistry (Saunders *et al*. 1997, 1998).

Most studies on the expression of ERβ in the human have used RNA techniques such as RT-PCR, RNase protection assays and *in situ* hybridisation (Byers *et al*. 1997, Kuiper *et al*. 1997). The first two methods are powerful tools to describe the presence of a particular gene in a tissue; however, they do not give a good indication of the type of cell that expresses the gene of interest. *In situ* hybridisation overcomes the problem of cellular localisation, but the technique is often difficult and the physiological relevance of the data is often difficult to interpret, because the expression of a particular mRNA does not always correspond with the expression of the functional protein. Immunohistochemistry overcomes the problem of identifying the precise cellular

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localisation of a particular mRNA does not always correspond with the expression of the functional protein. Immunohistochemistry overcomes the problem of identifying the precise cellular localisation of a, presumably, functional protein product. Although comprehensive and detailed immunohistochemical localisation of ERβ in the rat has recently been reported (Sar & Welsch 1999), there is no comprehensive report on the localisation of ERβ protein in the human, only studies of selected tissues (Van Pelt *et al*. 1999).

The present study was undertaken to identify those tissues in the human that express ERβ protein, to compare these results with those of the rat and to identify human tissues that might respond to oestrogen via ERβ rather than ERα. Additionally, the pattern of ERβ expression was compared with the pattern of ERα expression.

ERα and ERβ were successfully immunolocalised to several tissues in both male and female samples. The highest degree of ERβ nuclear staining was found in the ovary, breast and uterus, but differed from the pattern of ERα staining. Male reproductive tissue showed expression of ERβ in most tissues, with highest expression in the prostate, an ERα-negative tissue. In other organs and structures, ERα and ERβ were observed but not always in the same cell type. Some human cells produce ERβ but do not produce ERα, suggesting that oestrogen action in some human tissues may be mediated via the activation of ERβ rather than ERα.

MATERIALS AND METHODS

Antibodies

The monoclonal mouse anti-bovine ERα (05–394) antibodies directed against SDS-solubilised calf uterus ERα, and polyclonal rabbit anti-rat ERβ (06–629) antibodies developed against the N-terminal region of the human ERβ sequence were purchased from Upstate Biotechnology, Lake Placid, NY, USA. An additional polyclonal rabbit anti-rat ERβ (310) antiserum developed against the C-terminal region of the human ERβ sequence was purchased from Affinity Bioreagents Inc., Golden, CO, USA. The avidin–biotin blocking solution kit was from Vector Laboratories, Peterborough, Cambs, UK.

Immunohistochemistry

Normal human tissue samples obtained from adult human cadavers *post mortem* or from patients at the time of surgery for various pathological conditions were fixed in formol saline at the optimal rate of 1 cm^3/15·6 h (Polak & Van Noorden 1997) or for a fixed period of 24 h before processing into paraffin wax. Additional archival specimens were obtained from the Pathology Department at Leicester Royal Infirmary, Leicester, UK. Sections (4 μm) were mounted onto silane-coated slides and allowed to dry at 37 °C for 48 h. Samples were de-waxed, rehydrated and endogenous peroxidase activity quenched using hydrogen peroxide (6% v/v). After washing in double-distilled water for 5 min, sections were subjected to microwave antigen retrieval in 0·01 M citrate buffer, pH 6·0, for 30 min at 750 Watts power. Sections were allowed to cool undisturbed to approximately 37 °C over the next 30 min, washed (5 min each) in de-ionised H_2O and then in PBS–Tween 20 (0·05% v/v), and were blocked for 1 h with PBS containing 1% BSA. Sections were further blocked with normal swine serum or normal rabbit serum for 1 h for the detection of ERα or ERβ respectively. The sections were further blocked with avidin–biotin blocking solution according to the manufacturer's instructions. Excess liquid was removed from around the section and the slide was incubated with anti-ERα or anti-ERβ (1:50) in a humidified chamber for 18 h at 4 °C. After washing in PBS-Tween 20, sections were incubated with biotinylated swine anti-rabbit (ERβ) or biotinylated rabbit anti-mouse (ERα) immunoglobulins (Dako, Glostrup, Denmark) diluted 1:400 in PBS for 30 min. After a further wash in PBS-Tween 20 (30 min), the sections were incubated with horseradish-peroxidase avidin–biotin complex (Vector) for 30 min. After an additional wash in PBS–Tween 20 (30 min), bound antibodies were visualised with 0·05% diaminobenzadine (DAB) in 0·05 M Tris–HCl, pH 7·4, and 0·01% hydrogen peroxide, according to the supplier's instructions (Vector Elite kit). Sections were then washed in running tap water for 5 min, submerged in $CuSO_4$/NaCl solution (5 min), re-washed in tap water, dehydrated through graded alcohol, cleared with xylene and permanently mounted using XAM mounting medium (BDH, Poole, Dorset, UK). Specificity of immunostaining was confirmed using either pre-immune rabbit serum or purified mouse immunoglobulin G (IgG), as appropriate. Additionally, several control sections were produced by omission of the primary antibody or by incubation of anti-ERβ with 500 μM immunising peptide (a gift from S Boyd, Upstate Biotechnology or Affinity Bioreagents Inc.) or control protein (BSA) for 20 min at 37 °C prior to immunodetection. Images were captured on Fujichrome tungsten slide film using a Zeiss Axioplan microscope. All images are representative of at least two samples, analysed at least twice.

RESULTS AND DISCUSSION

Using polyclonal antisera raised against specific peptides localised at the N-terminal and C-terminal ends of the human ERβ sequence, ERβ was immunolocalised to cell nuclei in multiple tissues (Table 1). The pattern of expression for both antibodies was identical in all tissues studied (see examples in Figs 1–3). The C-terminal antibody has recently been used to show the immunolocalisation of ERβ in the female rat brain (Li *et al*. 1997, Alves *et al*. 1998). The monoclonal ERα showed specific staining in tissues previously reported to be positive for ERα, such as breast, vagina and ovary (Fig. 1). ERα was weakly expressed in ovarian granulosa and luteal cells (Fig. 1A) and differed in its level of expression compared with ERβ (Fig. 1B to K).

The highest level of ERβ expression was found in the nuclei of granulosa cells of small, medium and large ovarian follicles (Fig. 1B to G). ERβ immuroeactivity to ovarian granulosa cells could be inhibited by pre-incubation of the C-terminal antibody with 500 μM immunising peptide but not with BSA (Fig. 1C and B respectively). Additionally, staining results with the N-terminal (Fig. 1D) and C-terminal (Fig. 1E) were comparable. Similarly, the ERβ immunoreactivity to ovarian granulosa cells could be inhibited by pre-incubation of the N-terminal antibody with excess immunising peptide but not with excess C-terminal immunising peptide (Fig. 1F and G respectively). ERα was detectable in the stroma of the ovary, but not in the corpus luteum (Fig. 1H), whereas ERβ was found in the ovarian stroma and corpus luteum using both the N-terminal and C-terminal antibodies (Fig. 1I and J). Additional staining was also observed in primordial follicles (Fig. 1K).

These data are consistent with the results of *in situ* hybridisations (Byers *et al*. 1997, Kuiper *et al*. 1997) and with ERβ expression in rat (Saunders *et al*. 1997) and bovine (Rosenfeld *et al*. 1999) ovaries. The nuclei of human corpora lutea and corpora albicans showed significant levels of ERβ expression but, contrary to studies in the rat, some staining of cytoplasmic ERβ was found in both the human corpora lutea and corpora albicans (data not shown). ERα was also found in the nuclei of granulosa cells and ovarian stroma but not in corpora lutea/albicans, and ERα levels were lower, which is consistent with the studies of Byers *et al*. (1997) who found significantly lower expression of ERα mRNA compared with ERβ mRNA. Specific nuclear staining was almost completely abolished by incubation of the primary antibody with the peptide used for immunisation (Fig. 1C and F), but not with an unrelated peptide (BSA) (Fig. 1B and G). Other tissues of the female reproductive tract showed specific ERβ expression – Fallopian tube, uterus, cervix and vagina – with most of the expression confined to nuclear staining of epithelial cells (Fig. 1L to S). Some diffusion of staining to the cytoplasm of several epithelial cell types was observed (Fig. 1L, Q and S) and in the squamous epithelial cell layer of the vaginal wall (Fig. 1P). Endothelial cells in capillaries (Fig. 1N) and major blood vessels of all tissues studied showed only nuclear ERβ staining. These data suggest that cells in some tissues express only a nuclear ERβ receptor and other cells produce both nuclear and cytoplasmic forms of the beta-receptor. Because staining with both N-terminal and C-terminal antibodies was identical, the truncated ERβ isoforms recently reported (Moore *et al*. 1998, Ogawa *et al*. 1998*a*) are unlikely to be expressed, because a higher staining pattern with the N-terminal antibody would be predicted. There is currently little evidence that the truncated ERβ isoforms have any physiological relevance, except using *in vitro* reporter assays (Ogawa *et al*. 1998*b*), where data indicate that the truncated isoforms may inhibit activation of the full-length ERβ isoform. We observed a decrease in ERβ staining in the transition of resting to proliferative human breast that may support this notion.

Increased ERβ immunoreactivity was noted in the glands of normal resting breast when compared with the glands of proliferating breast (Fig. 1Q and R). Some ERβ staining was found in the cytoplasm of the resting breast epithelium, but both cytoplasmic and nuclear ERβ epithelial staining was reduced in the proliferating breast. ERβ was also present in the resting breast stroma, but almost absent in the proliferating breast. Recent studies have highlighted the fact that ERβ may be very important in breast tumorigenesis (Hu *et al*. 1998, Leygue *et al*. 1998, Vladusic *et al*. 1998), with an apparent increase in ERβ transcription during the hyperplastic phase. However, the apparent increase in ERβ mRNA levels could be attributed to an increase in cell number and not to a direct increase in transcriptional rates for ERβ. Indeed, our data suggest that the opposite is true. During activation of the normal breast, the ERβ protein concentration decreases, but there is an apparent increase in the number of ERβ-positive cells. This observation may explain the apparent ERβ mRNA increase reported in transformed MCF-7 cells (Hu *et al*. 1998). The protein concentration per cell is presumably dependent upon the stability of ERβ mRNA, which is currently unknown in breast tissue. Alternatively, during the proliferative process, ERβ transcription increases, but ERβ translation decreases.

TABLE 1. Immunohistological distribution of ERα and ERβ in adult human tissues

Tissue	ERα	ERβ
Central nervous system		
Cerebral cortex		
Neurons	−	+
Oligodendrocytes	+	+
Neurophil	−	−
Glial cells	+	+
Cerebellum		
Glial cells in molecular layer	−	+
Small nerve cells in granular layer	−	+
Purkinje cells	+	−
Hippocampus	+	−
Medulla oblongata		
Ependymal cells	−	+
Pons	+	+
Heart and vasculature		
Heart		
Myocardium	+	+
Endocardium	−	−
Epicardium	−	−
Purkinje fibres	+	−
Vessels		
Aorta	+	+
Coronary artery	+	+
Carotid artery	+	+
Inferior vena cava	+	+
Respiratory system and alimentary canal		
Lung (bronchiole)		
Columnar epithelium	−	+
Intermediate cells	−	+
Basal cells	+	+
Smooth muscle cells	+	+
Oesophagus		
Stratified squamous epithelium	+	+
Oesophageal mucous glands	−	+
Liver		
Hepatocytes	+	−
Kupfer cells	−	−
Venules	−	−
Bile duct	−	−
Hepatic portal vein	+	+
Stomach		
Villus epithelial cells	−	+
Goblet cells	+	−
Endocrine glands	−	−
Small intestine		
Villus epithelial cells	−	+
Goblet cells	+	−
Endocrine glands	−	−
Brunner glands	−	−
Large intestine		
Tubular glands	−	+
Intestinal glands	+	−
Sub-mucosal glands	+	+
Rectum		
Epithelial cells	−	+
Stromal cells	+	+
Endocrine system		
Pituitary		
Anterior	+	+
Posterior	+	+
Thyroid		
Follicular epithelial cells (thyrocytes)	−	+
Thyroid C-cells	−	+
Thyroid *continued*		
Fibroblasts	+	+
Endothelial cells	+	+
Adrenal		
Zona glomerulosa	−	+
Zona reticularis	−	+
Zona fasciculata	+	+
Chromaffin cells	+	+
Ovary		
Granulosa cells	+	+
Thecal cells	+	+
Stromal cells	+	+
Germinal epithelial cells	+	+
Corpora luteal cells	+	+*
Urinary system		
Kidney		
Collecting ducts	−	+
Loops of Henlé	−	−
Interstitial cells	+	−
Bowman's capsule	−	−
Glomerulus	−	−
Bladder		
Epithelial cells	+	+
Smooth muscle cells	+	+
Male reproductive system		
Testis		
Sertoli cells	+	+
Leydig cells	+	+
Epididymis	−	+
Vas deferens	−	+
Prostate		
Fibrocollagenous stroma	−	+*
Urethral epithelial cells	−	+*
Mucosal gland epithelial cells	−	+*
Female reproductive system		
Vagina		
Epithelial layer	+	+*
Connective tissue layer	−	+
Infiltrating lymphocytes	−	+
Uterus		
Endometrium luminal epithelia	+*	+*
Endometrium glandular epithelia	+	−
Endometrium stroma	+	+
Myometrium	+	+
Cervix		
Epithelial layer	+	+*
Connective tissue layer	+	+
Endothelial cells	−	+
Fallopian tubes		
Ciliated epithelium	+	+*
Peg cells	+	−
Connective tissue	+	+
Resting breast		
Adipocytes	+	+
Myoepithelial cells	+	+
Loose connective tissue	+	+
Active breast		
Adipocytes	+	+
Myoepithelial cells	+	+
Loose connective tissue	+	+
Lymphocytes (?)	−	+

(−) No immunoreactive staining observed; (+) positive immunoreactivity in the nucleus; (*) cytoplasmic staining present.

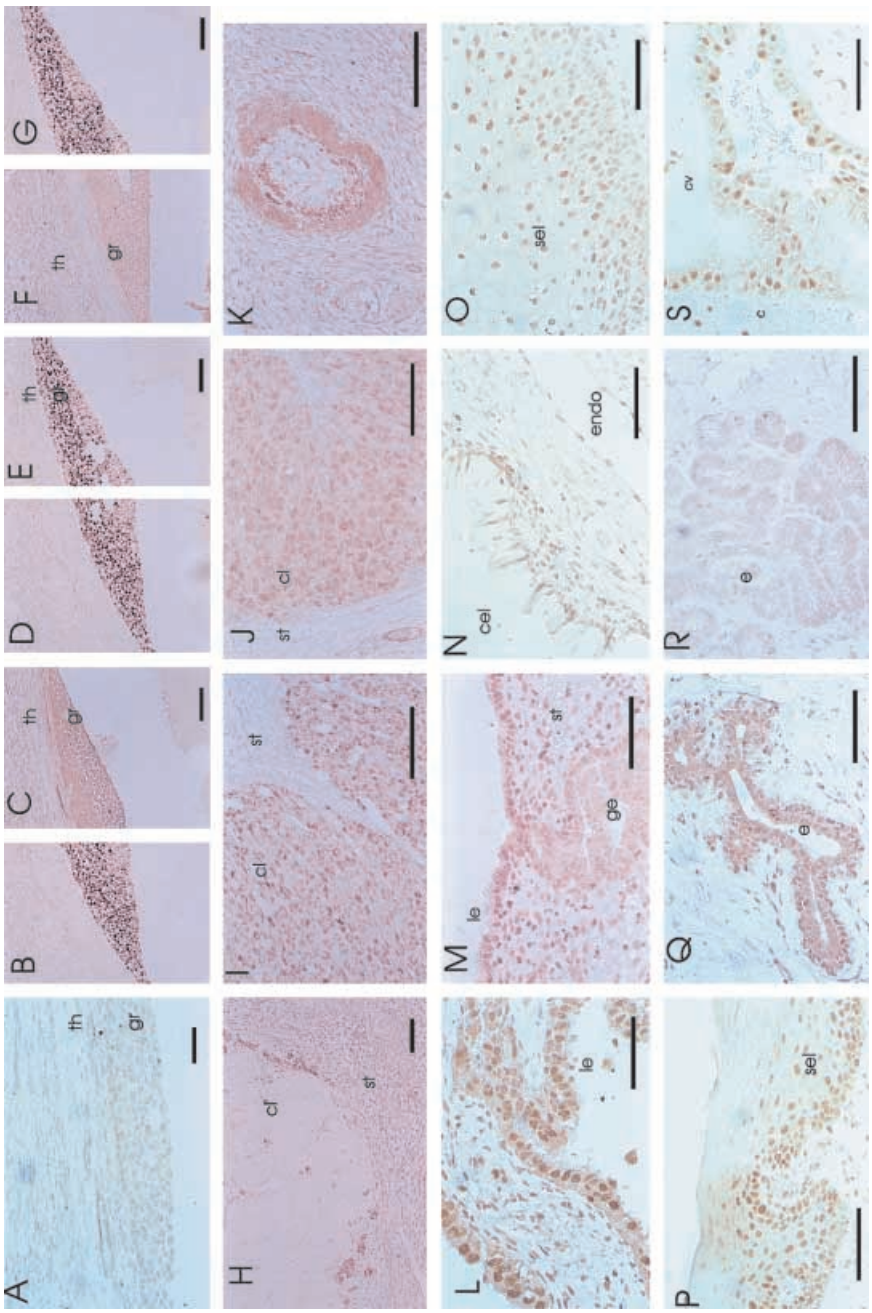

FIGURE 1. Immunohistochemical localisation of ERβ to adult female reproductive tissues. All samples were incubated with diluted rabbit IgGs at 1:50. ERα was localised to the nuclei of granulosa cells (gr), thecal cells (th) and stromal cells (A). Polyclonal ERβ immunoreactivity was localised to the nuclei of granulosa cells (gr), some thecal cells (th) and stroma (B–G). Specificity of the N-terminal antiserum was assessed by pre-absorption with irrelevant protein (B), or 500 μM immunising peptide prior to incubation (C) with a serial section of the same ovary, leading to loss of specific nuclear staining. Immunoreactive staining for N-terminal (D) and C-terminal (E) antibodies was identical. Specificity of the C-terminal antiserum was assessed by pre-absorption with 500 μM immunising peptide prior to incubation (F), or irrelevant protein (G). ERα was found in the nuclei of ovarian stromal cells (st), but not corpus luteum (cl) (H). N-terminal (I) and C-terminal (J) ERβ antibody was immunolocalised to the cytoplasm of the corpus luteum. Primary ovarian follicle (K) also showed ERβ immunoreactivity (N-terminal antibody). Luminal epithelial cells (le) in Fallopian tube (L), uterine endometrium (M), ciliated cervix glandular epithelial (cel) cells and endothelial cells (endo) (N), cervical squamous epithelial (sel) cells (O) and vaginal squamous epithelial cells (P) showed specific nuclear ERβ staining. Endometrial glands (ge) show a gradual decrease in ERβ staining intensity from high levels for epithelial cells near the lumen to absent at the base of the gland (M). Stromal cells in Fallopian tube (L), endometrium (M), cervix (N) and vagina (P) were all ERβ positive. ERβ is present in the nuclei of glandular components and some stromal cells of the inactive (resting) breast, (Q) and proliferative breast (R). Note the marked decrease in staining intensity for ERβ in the myoepithelial cells (e) of the proliferating breast compared with the inactive breast (R). ERβ was also found in the chorionic villus (cv) of the mature placenta (S) but not in the erythrocytes of an adjacent capillary (c). Each image is a representative of at least two samples, except the breast and endometrial samples where $n=7$ of each, and the ovary where $n=12$. Bars = 50 μm.

We also observed ERβ protein staining in adipose tissues, including that of the breast, confirming that breast adipose ERβ mRNA (Crandell *et al*. 1998) is probably translated into functional protein. What physiological role, if any, ERβ has in the breast remains unknown, but, predictably, it is an area for further investigation.

The uterus showed nuclear expression of both ERα and ERβ within the stroma of the endometrium, luminal epithelial cells and the entire myometrium. Expression of ERβ was absent from nuclei of most glandular epithelial cells (Fig. 1M) with some diffuse cytoplasmic staining detectable in some glandular epithelial cells at the neck of the gland (Fig. 1M), suggesting the presence of ERβ in these cells. The nuclei of luminal epithelial cells were rich in ERβ, but at the interface between luminal epithelial cells and glandular epithelial cells expression appears to diminish and lessen deeper into the gland (Fig. 1M). In contrast to the glandular epithelial cells, ERβ was found in the nuclei of all stromal cells (Fig. 1M). ERα, by contrast, was found in the nuclei of luminal epithelial cells, glandular epithelial cells and stromal cells (data not shown), which is consistent with the work of others (Lessey *et al*. 1988, Amso *et al*. 1994, Tibbetts *et al*. 1998, Matsuzaki *et al*. 1999).

The pattern of ERα expression in the human endometrium differs from that reported in the rat, where nuclear ERα was found in many epithelial cell types but no cytoplasmic stain was observed (Saunders *et al*. 1997). The significance of this differential ERα expression between the rat and human uterus is unclear but may have implications on the use of rodents in the study of the growth-stimulatory response of the uterus to oestrogens. The lack of ERβ expression in the glandular epithelial cells of the human uterus may reflect the specimens used. The samples were obtained from women in the late secretory phase of the menstrual cycle. It is possible that the ERβ status of the human glandular epithelial cells may change during the menstrual cycle, as a recent report suggests, with the levels of ERβ changing from 'very strong' for proliferative endometria to 'very weak' for secretory endometria (Matsuzaki *et al*. 1999). Additionally, these authors report the presence of ERβ mRNA in stromal cells but at a lower level compared with glandular epithelial cells, and that stromal ERβ mRNA levels remain constant throughout the menstrual cycle. We have examined more than 30 endometrial specimens and have not been able to detect ERβ in the glandular epithelial cells of proliferative or secretory endometria. This suggests that although these cells may contain ERβ mRNA, the protein is not expressed and that ERβ may have no physiological role in the endometrial glandular epithelial cells. Alternatively, it is also possible that ERβ expression in glandular epithelial cells is regulated by interactions with other members of the steroid receptor superfamily in a temporal manner, just as progesterone receptor is regulated by ERα and retinoic acid receptor (Savouret *et al*. 1994).

The male urogenital system showed immunoreactive staining for both ERα (Fig. 2A and Table 1) and ERβ (Fig. 2B to K and Table 1). Staining was confined to nuclei of mostly luminal epithelial cells of many tissues except the epididymis (Fig. 2L) where extensive cytoplasmic staining was visible. As expected, high levels of ERβ were found in the prostate with both the N-terminal antibody (Fig. 2B) and the C-terminal antibody (Fig. 2C and D). ERβ was also localised to collecting duct tubules of the renal medulla (Fig. 2F and G), but, significantly, not to renal glomeruli or renal cortical tubules (Fig. 2E). Loops of Henlé and capillaries were also negative for ERβ and, interestingly, ERβ was consistently localised to the basal surface of epithelial cells lining the collecting ducts (Fig. 2G). The bladder (Fig. 2H) showed specific ERβ staining in the nuclei of epithelial and smooth muscle cells, and the epididymis showed staining in basal cells, principal cells, smooth muscle cells and cells that constitute the connective tissue layer (Fig. 2I). Epithelial cells and smooth muscle cells of the ductus deferens also showed specific ERβ staining (data not shown). A high level of nuclear ERβ staining was observed with both the N-terminal (Fig. 2J) and the C-terminal (Fig. 2K) antibodies. Spermatocytes, Sertoli cells, interstitial cells and spermatogonia all showed specific ERβ immunoreactivity.

The staining pattern for ERβ in the human male urogenital tract was associated with many cell types involved in semen development and secretion. In particular, the staining pattern in the testis and prostate was similar to that seen in the adult male rat (Saunders *et al*. 1997, 1998). However, the finding of specific ERβ staining to the basal surface of kidney collecting duct tubules, but not to the cytoplasm or the nucleus (Fig. 2F and G) is difficult to explain. Historically, the human kidney has been thought to be unreactive to oestrogen stimulation, although renal carcinogenesis is inducible with oestrogens in the rodent (Bhat *et al*. 1993). Therefore, the presence of immunoreactive ERα and ERβ in the human kidney is surprising. However, recent data (Hardy *et al*. 1999) suggest that oestradiol can interact on the basal surface of proximal tubule cells to increase kidney androgen-regulated protein (KAP), suggesting the possibility of an ERβ-dependent effect in the human kidney.

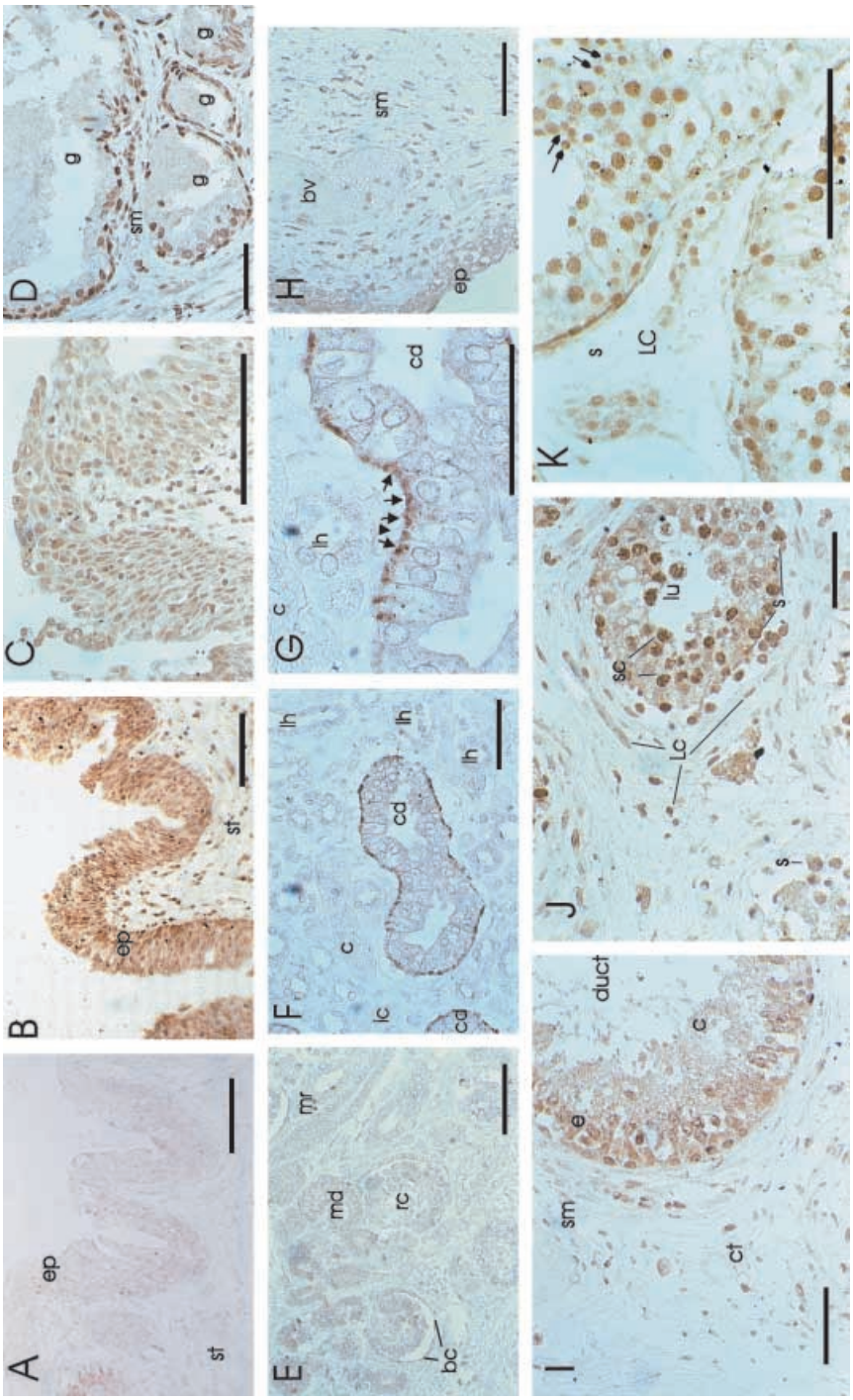

FIGURE 2. ERα and ERβ immunolocalisation to adult male urogenital tract tissues. All samples were incubated with diluted IgGs at 1:50. ERα was absent from the prostate (A), whereas ERβ was localised to the nuclei of epithelial (ep) and stromal (st) cells of the ventral surface (N-terminal (B); C-terminal (C) antibodies) of the prostate. (D) ERβ was also localised to the nuclei of epithelial and smooth muscle (sm) cells within the glandular (g) region the prostate. (E) The renal cortex was negative for ERβ; Bowman's capsule cells (bc), the macula densa (md), renal corpuscle (rc) and adjacent medullary rays (mr) were all negative. (F) Collecting ducts (cd) in the renal medulla were ERβ positive, whereas Loops of Henlé (lh), interstitial cells (ic) and erythrocytes in capillaries (c) were negative. (G) The ERβ observed in renal medullary collecting ducts (cd) was confined to the basal surface (arrows). (H) Bladder epithelial (ep), smooth muscle (sm) and isolated mononuclear immune cells of an adjacent blood vessel (bv) showed ERβ staining. (I) Smooth muscle (sm) and connective tissue cells (ct) in the body of the epididymis showed nuclear ERβ staining, whereas the epithelium (e) lining the duct showed both nuclear and cytoplasmic staining. Clumps of spermatozoa (c) exhibited non-specific stain. (J) N-terminal ERβ staining of testicular Sertoli cells (s), luminal (lu), spermatocytes (sc) and interstitial cells (Lc). (K) C-terminal ERβ staining of Sertoli cells, interstitial cells (LC) and spermatogonia (arrows). Each image is a representative of at least two samples. Bars = 100 μm.

ERα was found in both renal medullae and cortexes, but isolated to the nuclei of cells constituting the Loop of Henlé and renal corpuscle (data not shown). ERα was absent from the collecting ducts, suggesting that oestrogen-dependent effects in these cells occurs only through ERβ-dependent mechanisms. Additionally, the intensity of ERβ staining in the male samples was more intense than that of ERα (data not shown), suggesting that a high estrogen environment may affect ERβ expression in some tissues, as has been suggested by others (Chu & Fuller 1997).

ERβ expression was not confined to the female and male reproductive tracts. The oesophagus, lung, small intestine, heart, many areas of the brain, thyroid, stomach, intestine, rectum, smooth muscle cells and endothelial cells of blood vessels were all positive for ERβ (Table 1 and Fig. 3). Both ER isoforms were found in the nuclei of cells of the tissues described (see Table 1), with the exception of hepatocytes and the hippocampus where only ERα was observed. Although ERβ has previously been demonstrated in the rat hippocampus (Li *et al*. 1997), we were unable to detect significant ERβ staining in the human hippocampus (Fig. 3J). ERα was detectable in human hippocampus (Fig. 3I) but ERβ was not considered to be present, even though some very weak staining could be seen (Fig. 3J). These data suggest that ERβ is not expressed in the human hippocampus, or that the antigen retrieval method used in the present study was insufficient to unmask the formalin-induced cross-links. In the original use of the C-terminal antibody in the rat (Li *et al*. 1997), the hippocampus was perfused with 2% acrolein and used as cryosections. Therefore, it follows that some of the cells and tissues reported herein to be negative for ERβ may be proved to be immunopositive under different fixation/unmasking conditions.

Although the distribution of the ERβ isoform appears to be closely related to the expression of ERα in most tissues (Table 1), ERβ expression does not appear to be linked to ERα expression. Some ERα-positive cells lack ERβ and *vice versa* (see Table 1), raising the possibility that there are distinct ERα- and ERβ-dependent transcriptional pathways. For example, the prostate lacks ERα but contains ERβ, leading to the conclusion that the clinical treatment of the prostate for prostatic cancer (Carlstrom *et al*. 1997) can be mediated only through an ERβ-dependent pathway.

The data presented are qualitatively similar to those found for the rat (Saunders *et al*. 1997), with the minor exceptions stated above. Additionally, the pattern of ERβ protein expression in most human tissues is similar to the pattern of ERβ transcript expression. For example, ERβ protein was detected in tissues shown to have ERβ transcript such as lung and adrenal (Kuiper *et al*.1997), heart (Grohe *et al*. 1998), rat forebrain (Shughrue *et al*. 1997, Osterlund *et al*. 1998) and aorta. In the aorta, ERβ may have an important regulatory role since it has recently been shown to increase in rats subjected to experimental injury (Lindner *et al*. 1998).

To our knowledge, this is the first report to indicate that ERβ protein is expressed in the adult human thyroid, gastrointestinal tract and renal collecting duct tubules, although ERβ transcripts were found in the kidney of the mid-gestational human fetus (Brandenberger *et al*. 1997).

The availability of specific antisera to ERβ will aid the investigation of this novel protein in the human. These antisera complement the RT-PCR and RNase-protection assays performed elsewhere, but, as detailed in the Introduction, RNA techniques only measure relative levels of RNA expression in an entire tissue homogenate, whereas this study and that of Saunders *et al*. (1997) establish the precise cellular localisation of the protein. A limited number of studies using *in situ* hybridisation has been used to identify cellular expression of ERβ in the ovary (Byers *et al*. 1997), testis (Couse *et al*. 1997*b*) and brain (Shugrue *et al*. 1996, Register *et al*. 1998, Shugrue 1998). However, the data reported previously are contradicted by some of the data presented here and by the rat ERβ immunolocalisation study (Saunders *et al*. 1997). For example, in Byer's *in situ* hybridisation study (Byers *et al*. 1997), ERβ mRNA was undetectable in the corpus luteum of the normal rat ovary, yet ERβ protein is readily detectable in the rat ovary (Saunders *et al*. 1997). We also observed ERβ protein in the human corpus luteum (see Fig. 1F and G) and in the nuclei of ovarian surface epithelia, confirming previous findings (Saunders *et al*. 1997, Hillier *et al*. 1998).

This study extends all the preceding body of work by examining the immunological localisation of ERβ in human tissues not studied by previous authors. However, there are other discrepancies between our study and that of others (Couse *et al*. 1997*a*, Saunders *et al*. 1997). Although we have shown ERβ to be expressed in the uterus, we could not detect ERβ in the endometrial glandular epithelial cells. This disagrees with the original data presented by Couse *et al*. (1997*a*), who reported the absence of ERβ RNA in the uterus, suggesting that ERβ was not expressed in the uterus. Later, the same group reported ERβ to be weakly expressed (Couse *et al*. 1997*b*). However, there is no indication in their study which cells were weakly expressing ERβ.

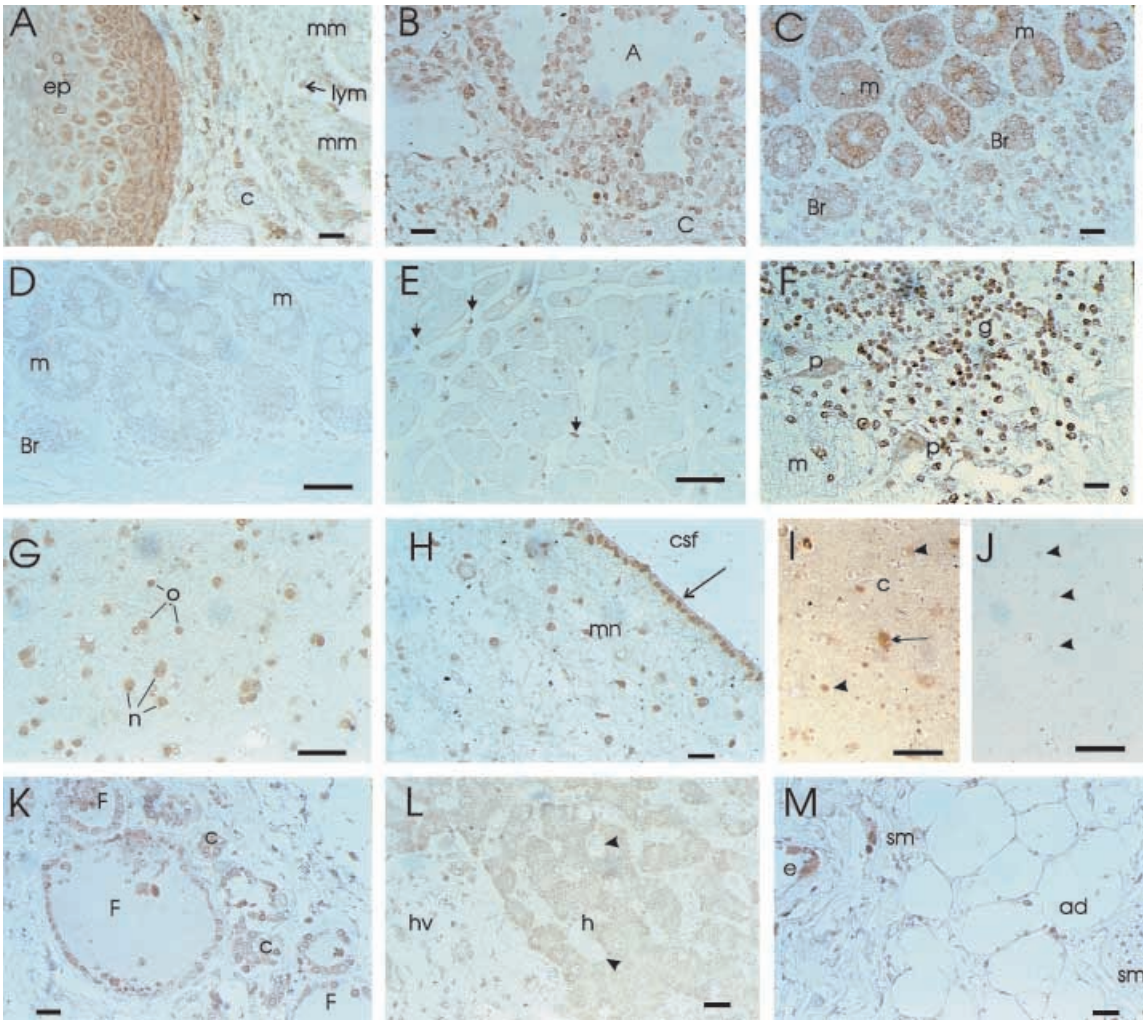

FIGURE 3. Immunohistochemical localisation of ERβ to multiple human tissues. (A) ERβ staining using the N-terminal ERβ polyclonal antibody was localised to nuclei of epithelial cells (ep), cells in the muscularis mucosae (mm), mononuclear immune cells probably infiltrating lymphocytes (lym) but not capillaries (c) within the oesophagus. (B) Lung surface epithelia of alveoli (A) were ERβ positive but erythrocytes within adjacent lung capillaries (lg) were not. (C) Cytoplasmic ERβ was observed in mucosal mucin-secreting epithelial cells (m) of the small intestine but not in Brunner's glands (Br) and (D) the stain could be blocked by prior incubation of antibody with recombinant full-length ERβ protein. (E) ERβ was also localised to the nuclei of cardiomyocytes and cardiac fibroblasts (arrows) of the heart ventricle. Most regions of the brain (F-J) including cells in the granular layer (g), but not Purkinje cells (p) or cells of the molecular layer (m), within the cerebellum (F), oligodendrocytes (o), and neurons (n) in the cerebral cortex (G), and motor neurons (mn) and epithelial cells (arrow) lining the cerebrospinal fluid space (csf) within the pons (H) were ERβ positive. The hippocampus was positive for ERα (I) showing specific staining in oligodendrocytes (arrowheads), neuronal cell bodies (arrows) but not capillaries (c) and very weak staining for ERβ was observed in oligodendrocytes (arrowheads) (J). Thyroid follicular epithelial cells (F) and thyroid C-cells (c) were positively stained for ERβ (K). Although a weak cytoplasmic stain for ERβ was observed in hepatocytes (h) surrounding sinusoidal channels (arrowheads) of the adult liver (L), it was not specific, although vascular cells in the hepatic vein (hv) were positively stained. All cells in the aorta (M) showed nuclear localisation of ERβ, including endothelial cells (e), smooth muscle cells (sm) and adipocytes (ad). Bars=100 μm.

The cellular distribution of ERβ in the human uterus also shows minor discrepancies when compared with its distribution in the rat uterus. The rat shows ERβ expression in all cell types, whereas ERβ expression was undetectable in human endometrial glandular epithelial cells. However, the rat uteri were removed on the day of pro-oestrus, whereas our samples were removed at 7–10 days post-ovulation, suggesting that ERβ may be differentially expressed throughout the menstrual cycle. However, although *in situ* hybridization studies suggest that ERβ is probably down-regulated towards the end of the menstrual cycle (Matsuzaki *et al*. 1999), because the staining was weak, the authors may be over-interpreting their data. This is another area that needs clarification.

In the rat immunolocalisation study, ERα was undetectable in rat ovarian granulosa cells. We observed specific staining for ERα in human ovarian granulosa cells (Table 1), as did others (Revelli *et al*. 1996). The reason for the discrepancy between the immunolocalisation of rat and human ERα in human granulosa cells is unknown, but may relate to the methods used to prepare tissues prior to immunohistochemistry. We have found that steroid receptor proteins are susceptible to loss if tissue is not fixed in formol saline at the optimal rate of 1 cm^3/15·6 h (authors' unpublished observations), as described by others (Taylor *et al*. 1994). Additionally, it is possible that the ERα antibodies used in our study are better suited for immunohistochemical techniques. Certainly, there are anti-rat ERα antibodies that function well in immunoblotting procedures but not in immunohistochemical methods, and *vice versa* (Fisher *et al*. 1997). The anti-bovine ERα antibody (05–394) that we used in this study is useful in both immunohistochemistry and immunoblotting methods. Cells of oestrogen-responsive tissues (e.g. the female reproductive tract, breast, etc.) showed intense nuclear ERα staining, as expected (Table 1), indicating the specificity and usefulness of the ERα antibody used for the present study.

Future work will assess the expression of these nuclear receptors in the uterus during the menstrual cycle and look for potential mechanisms that control their expression.

In conclusion, the recent discovery of ERβ means that the potential sites of oestrogen action may have to be re-evaluated. This paper points at potential starting points for investigation in the human, and at potential discrepancies between rodents and man. This latter point may become more important in the interpretation of many experiments using exogenous and synthetic oestrogen.

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