Expression and regulation of oestrogen receptors in the human corpus luteum

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Abstract

The molecular mechanisms underlying the control of corpus luteum lifespan in women are not fully understood. Oestradiol has various luteolytic, or luteotrophic, functions in some species, and as it is synthesised within the human corpus luteum, it is an excellent candidate molecule to be a paracrine regulator of luteal function. This study aimed to comprehensively investigate the expression, regulation and effects of oestrogen receptors (ER) in human luteal cells. Genomic oestrogen receptors ER α , ER β 1 and ER β 2 were immunolocalised in human corpora lutea from throughout the luteal phase. mRNA expression was investigated throughout the luteal phase and after luteal rescue with exogenous human chorionic gonadotrophin (hCG). The regulation of ER expression and oestradiol action was investigated in cultures of luteinised granulosa cells. ER subtypes ER β 1 and ER β 2 were localised throughout the luteal phase to steroidogenic cells in the human corpus luteum and cells of the surrounding stroma. Unlike follicular granulosa cells, steroidogenic cells in the corpus luteum showed minimal ER α immunostaining. The presence of endothelial cells in the granulosa cell layer with ER β 1 and ER β 2 positive nuclei was noted. ER β 1 and ER β 2 were differentially regulated across the luteal phase with ER β 1 maximally expressed in the mid-luteal phase, while ER β 2 expression was maximal in the early luteal phase. *In vivo* and *in vitro*, hCG had no long-term effect on ER expression, although *in vitro* hCG and oestradiol acutely down-regulated ERs. Treatment with oestradiol *in vitro* down-regulated 11 β -hydroxysteroid dehydrogenase type 1 and inhibin β A subunit confirming a functional oestradiol response. These data highlight functional and differentially regulated oestradiol reception in human luteal cells.

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Introduction

The molecular regulation of luteolysis and maternal recognition of pregnancy in women is still not fully understood (Duncan 2000). In other species the understanding of luteolysis is more advanced. A common pattern is the release of a luteolytic factor from the uterus, prostaglandin F_{2 α} , which can be suppressed by the conceptus (McCracken *et al.* 1970, Hansel *et al.* 1973). This is not the case in women, as hysterectomy does not prolong the lifespan of the corpus luteum (Niswender *et al.* 2000). Luteolysis is also not initiated through endocrine changes in pituitary hormones. Maintaining the luteinising hormone (LH) pulse frequency during the luteal phase does not affect luteolysis (Hutchison *et al.* 1986). These data suggest that factors involved in luteolysis are locally produced and active within the corpus luteum itself.

In simulated early pregnancy, human chorionic gonadotrophin (hCG) has effects on immune cells, endothelial cells and fibroblasts (Duncan *et al.* 1998a, 1998b, Duncan 2000, Wulff *et al.* 2001) which do not

express LH/hCG receptors (LHCGR). Therefore, if paracrine molecules, which are regulated by hCG, are involved in luteolysis, these molecules should be the products of steroidogenic cells and have receptors on target cells. Indeed, using this approach we have recently identified activin A as a candidate paracrine regulator of tissue remodelling during luteolysis in women (Myers *et al.* 2007a).

We have also investigated progesterone as a locally produced molecule with receptors on various luteal cell types (Maybin & Duncan 2004). While progesterone is an attractive paracrine regulatory molecule in the corpus luteum (Vega & Devoto 1997, Stouffer 2003), characterisation of important and clear luteal effects of progesterone has remained elusive (Devoto *et al.* 2002a, 2002b). The same cannot be said for oestrogen. Oestrogen has a clear role in the regulation of luteal function in some species (Niswender *et al.* 2000).

Previously, it was thought that oestradiol did not have effects in the primate corpus luteum based on the lack of expression of oestrogen receptors (ER) in the corpus luteum of rhesus monkeys (Chandrasekher *et al.* 1994).

After the discovery of ER β , this was reinvestigated and ER β has been reported in the corpus luteum of marmoset monkeys and women (Saunders *et al.* 2000). Since then some studies have reported equal levels of ER α (*ESR1*) and ER β (*ESR2*) mRNA in the human corpus luteum (Misao *et al.* 1999) and others suggest that ER β might be dominantly expressed over ER α (Hosokawa *et al.* 2001). Although studies of ER α and ER β have been reported in the rat (Byers *et al.* 1997, Telleria *et al.* 1998, Sar & Welsch 1999) and rhesus monkey (Duffy *et al.* 2000) corpus luteum, and in human luteinised granulosa cells (Chiang *et al.* 2000), the differential regulation, cellular localisation and the role of ERs in human luteal cells remain uncertain.

This study therefore aims to (1) investigate the localisation and expression of ER α , ER β 1 and ER β 2 in human corpus luteum across the luteal phase; (2) investigate the cellular localisation of these receptors; (3) determine whether mRNA expression is regulated by hCG *in vivo* and *in vitro*; and (4) study whether oestradiol regulates the mRNA expression of key regulators of luteal function in cultures of luteinised granulosa cells *in vitro*.

Results

Localisation of ER subtypes in human ovary

Nuclear staining was observed for ER α (Fig. 1B), ER β 1 (Fig. 1C) and ER β 2 (Fig. 1D) in the human ovary, while no staining could be detected in negative control sections (Fig. 1A). ER β 1 and ER β 2 are both expressed in the nuclei of follicular and luteal granulosa cells (Fig. 1C and D

Figure 1 Immunolocalisation of oestrogen receptor subtypes in the human ovary. (A) Negative control section of human ovary showing a corpus luteum (CL), an antral follicle (FOL) and stromal tissue (STR). (B) Immunostaining (brown) for ER α demonstrates its protein localisation in the nuclei of follicular cells (arrowheads), while staining in the cells of the corpus luteum is negligible (arrows). (C) ER β 1 protein is expressed in the nuclei of cells of the corpus luteum (arrows) and in cells of the antral follicle (arrowheads). (D) Immunolocalisation for ER β 2 shows a stronger expression in the nuclei of follicular cells (arrowheads) when compared with the cells of the corpus luteum (arrows). Scale bar = 30 μ m.

respectively), although ER β 2 is more strongly expressed in the cells of the corpus luteum than in follicular cells (Fig. 1D). Both ER β 1 and ER β 2 show a strong expression in the nuclei of surrounding stromal cells and theca-lutein cells. Immunolocalisation of ER α showed that this receptor is expressed in the nuclei of granulosa cells of antral follicles, while ER α staining in the corpus luteum appeared negative or very weak (Fig. 1B). This suggests that the main receptor in the human corpus luteum is ER β and that ER α may be down-regulated in the corpus luteum when compared with the follicle.

Granulosa-lutein cells co-express ER β 1 and ER β 2

Using double immunofluorescence, we then investigated whether luteal steroidogenic cells had both ER β 1 and ER β 2 receptors. As shown in Fig. 2A and B, ER β 1 (green signal) seems to be the more predominant isoform localised to the nuclei of granulosa-lutein cells when compared with ER β 2 (red signal). Some cells show a co-localisation of ER β 1 and ER β 2 (orange signal), while some cells show only ER β 1 localisation (arrows) and others show a very weak co-localisation (arrowheads). These data suggest that these receptors co-exist in granulosa-lutein cells. While it is possible that some cells have mainly ER β 1, no cells with mainly ER β 2 were detected in any of the sections analysed across the luteal phase.

Figure 2 (A and B) Double immunofluorescence for ER β 1 (green) and ER β 2 (red) in a mid-luteal human corpus luteum. Co-localisation of ER β 1 and ER β 2 is shown as orange nuclei. Arrows demonstrate cells that only express ER β 1, while arrowheads demonstrate a very weak co-localisation of ER β 1 and ER β 2. (C) Co-localisation (arrows) in another mid-luteal human corpus luteum of ER β 1 (green) with CD31/PECAM-1 (red), a marker for endothelial cells. (D) Co-localisation (arrows) in the human corpus luteum of ER β 1 (green) with CD31/PECAM-1 (red). In all cases the scale bars represent 20 μ m.

ER β 1 and ER β 2 are localised to the nuclei of endothelial cells

It is clear that ER β 1 and ER β 2 can be localised to different cell types in the human corpus luteum. As the progesterone receptor in the corpus luteum is absent from endothelial cells (Maybin & Duncan 2004), we investigated whether these cells express ER β receptors. Dual immunofluorescence on the slides of the corpus luteum for ER β 1 or ER β 2 with CD31/PECAM-1, a well-described marker for endothelial cells (Cao *et al.* 2002) revealed that both ER β 1 and ER β 2 are expressed in the nuclei of endothelial cells (see arrows in Fig. 2C and D respectively) at all stages of the luteal phase. Thus, luteal microvascular endothelial cells have the capacity to directly respond to oestradiol.

Immunolocalisation of ER β 1 and ER β 2 across the luteal phase

Next, we analysed the possibility that expression of ER may vary throughout the luteal phase. Immunohistochemistry for ER β 1 and ER β 2 was analysed in early, mid- and late luteal phase corpora lutea (Fig. 3). Positive immunostaining for ER β 1 and ER β 2 was observed in the nuclei of both granulosa-lutein cells and theca-lutein cells, for all sections classified as either early, mid- or late luteal phase, based on cell morphology (Maybin & Duncan 2004). Positive immunostaining could also be seen throughout the luteal phase in some of the cells in

Figure 3 ER β 1 expression in human corpus luteum during (A) the early luteal, (B) mid-luteal and (C) late luteal stages of the luteal phase, and (D) ER β 2 expression in human corpus luteum during the early luteal, (E) mid-luteal and (F) late luteal phase. BV, blood vessel; GLC, granulosa-lutein cell; S, stroma and TLC, theca-lutein cell. Scale bars = 30 μ m.

the stromal areas (Fig. 3). Using the same immunostaining intensity scoring system that revealed a down-regulation of progesterone receptor in the late luteal phase (Duncan *et al.* 2005a), no differences were observed in ER β 1 or ER β 2 immunostaining during different stages of the luteal cycle in any cell compartment ($P < 0.05$, Kruskal–Wallis test; data not shown).

The effect of hCG on the expression of ER in human corpora lutea in vivo

In order to determine the effect of hCG on the luteal expression of ER in women, mRNA expression for ERs was examined in archival corpora lutea tissues collected from early, mid, late and rescue stages of the luteal phase. Although we could not immunolocalise ER α in the human corpus luteum (Fig. 1B), its mRNA is expressed at a low level. There were no changes across the luteal phase or after hCG exposure to mimic maternal recognition of pregnancy (Fig. 4A). There was, however, evidence of changing expression of the ER β receptors. ER β 1 mRNA expression was maximal in

Figure 4 (A–C) *In vivo* mRNA expression of oestrogen receptors in human corpora lutea during the luteal phase. (A) ER α , (B) ER β 1 and (C) ER β 2 expressions are shown in the early (LH + 1 to LH + 5), mid- (LH + 6 to LH + 10) and late (LH + 11 to LH + 14) luteal phases and after treatment with hCG from LH + 7 for 5–8 days (rescue). (D–F) *In vitro* chronic manipulation with hCG in luteinised granulosa cell cultures designed to mimic the luteal phase. Fresh cultures (day 2) were treated with low dose hCG until day 7, and then hCG was increased (day 13 + hCG) or removed (day 13 – hCG). (D) ER α , (E) ER β 1 and (F) ER β 2 expressions. (* $P < 0.05$; ** $P < 0.01$, Kruskal–Wallis test).

the mid-luteal phase (Fig. 4B) and luteal rescue reduced mRNA expression ($P < 0.05$, Kruskal–Wallis test) when compared with the mid-luteal phase. The pattern was different when *ERβ2* mRNA expression was investigated. It was maximal in the early luteal phase (Fig. 4C) and expression was significantly lower in the mid-luteal phase compared with the early luteal phase ($P < 0.01$, Kruskal–Wallis test). There were no differences after exposure to hCG *in vivo* to rescue the corpus luteum (Fig. 4C). These data suggest that *ERβ1* and *ERβ2* may be differentially regulated across the luteal phase.

Differential expression of ERs in the prolonged cultures of luteinised granulosa cells

As different cells express ERs in the human corpus luteum (Figs 1 and 3), we investigated granulosa-lutein cell expression separately using a novel model system of prolonged cultures of luteinised granulosa cells to mimic the human luteal phase and luteal rescue in the absence of other cell types (Duncan *et al.* 2005a). *In vitro*, *ERα* mRNA expression was decreased in prolonged cultures when hCG had been removed (Fig. 4D). The mRNA expression of *ERβ1* (Fig. 4E) was not significantly different in these *in vitro* cultures of luteinised granulosa cells mimicking the luteal phase ($P > 0.05$, Kruskal–Wallis test). In contrast, *ERβ2* mRNA expression (Fig. 4F) was reduced in 7-day cultures ($P < 0.05$, Kruskal–Wallis test) and 13-day cultures with hCG ($P < 0.01$, Kruskal–Wallis test) when compared with 2-day cultures.

HCG actively regulates ER expression in vitro

As it seems that ERs may be differentially regulated, we investigated whether hCG had differential effects on the mRNA expression of the different ER isoforms in the short term. The treatment of cultured human luteinised granulosa cells with 100 ng/ml hCG for 24 h resulted in a significant down-regulation of *ERα* ($P < 0.05$, *t*-test; Fig. 5A), *ERβ1* ($P < 0.05$, *t*-test; Fig. 5B) and *ERβ2* ($P < 0.05$, *t*-test; Fig. 5C) mRNA expression. This confirms that ERs can be regulated in luteal steroidogenic cells acutely, but suggests that the differential effects seen in *ERβ1* and *ERβ2* mRNA expression are not a result of acute LH/hCG signalling *per se*.

Luteinised granulosa cells have a functional oestrogen signalling pathway

Finally, we determined the functional effects of oestradiol on the cultures of luteinised granulosa cells *in vitro*. Using physiological concentrations (Andersen & Hornnes 1994), treatment with oestradiol (10^{-6} mol/l for 24 h) could down-regulate the mRNA expression of both *ERα* ($P < 0.01$, *t*-test; Fig. 5D) and *ERβ1* ($P < 0.05$,

Figure 5 (A–C) Short-term stimulation of luteinised granulosa cells with 100 ng/ml hCG for 24 h significantly down-regulated the mRNA expression of (A) *ERα*, (B) *ERβ1* and (C) *ERβ2*. (D–F) Short-term stimulation of luteinised granulosa cells with 10^{-6} mol/l oestradiol (E_2) for 24 h significantly down-regulated the mRNA expression of (D) *ERα* and (E) *ERβ1* although (F) the tendency for reduction in *ERβ2* mRNA expression was not significant (* $P < 0.05$; ** $P < 0.01$, *t*-test).

t-test; Fig. 5E). There appeared to be no effect on *ERβ2* ($P = 0.1090$, *t*-test; Fig. 5F).

Investigation of other important factors in the corpus luteum showed that oestradiol has no effect on the mRNA expression of *LHCGR* (Fig. 6A), *StAR* protein (*STAR*; Fig. 6B), vascular endothelial growth factor (*VEGF*; Fig. 6C) and connective tissue growth factor (*CTGF*; $P = 0.0976$, *t*-test; Fig. 6D) in luteinised granulosa cell cultures. However, oestradiol significantly down-regulates the mRNA expression of inhibin βA subunit (*INHBA*; $P < 0.05$, *t*-test; Fig. 6E) and 11 β -hydroxysteroid dehydrogenase type 1 (*HSD11B1*; $P < 0.05$, *t*-test; Fig. 6F) in these cultures.

Discussion

This study has investigated genomic ERs in the human corpus luteum across the luteal phase. The regulation and effects of these receptors have been further analysed using an established model of luteinised granulosa cell cultures *in vitro*. We have shown that, in contrast to follicular granulosa cells, granulosa-lutein cells tend to immunolocalise *ERβ* rather than *ERα*. Both *ERβ1* and

Figure 6 Short-term stimulation of luteinised granulosa cells with 10^{-6} mol/l oestradiol (E_2) for 24 h has no statistically significant effects on the mRNA expression of (A) LH/hCG-R (*LHCGR*), (B) *STAR*, (C) *VEGF* and (D) *CTGF*. (E) Inhibin βA subunit (*INHBA*) and (F) 11 β HSD type 1 (*HSD11B1*) were significantly down-regulated after stimulating luteinised granulosa cells with 10^{-6} mol/l oestradiol for 24 h (* $P < 0.05$, *t*-test).

ER β 2 are expressed in various luteal cell types across the luteal phase, including endothelial cells. The role of these receptors is not clear, but we provide evidence for a functional ER pathway in luteinised granulosa cells and evidence that *ER β 1* and *ER β 2* mRNA expression is regulated and that this regulation may be differential for each receptor type.

Previous investigation into ER expression in the corpora lutea of women produced some contradictory results. One study showed *ER α* and *ER β* transcripts to be co-expressed at similar levels (Misao *et al.* 1999), while another showed *ER α* protein in the follicles but not in the corpus luteum (Taylor & Al-Azzawi 2000). Further studies reported *ER β* protein in human luteal tissue (Saunders *et al.* 2000, Taylor & Al-Azzawi 2000). We have confirmed that human corpora lutea express mRNA for *ER α* and *ER β 1* and shown *ER β 2* expression. In addition, we have been able to immunolocalise *ER α* to the follicle but not the corpus luteum, whereas *ER β 1* and *ER β 2* could be immunolocalised to the corpus luteum. The human corpus luteum expresses both *ER β 1* and *ER β 2* message and protein.

There seems to be regulation across the normal luteal phase in the expression of these receptors. *ER β 1* mRNA is maximally expressed in the mid-luteal phase, while

ER β 2 mRNA expression is maximal as the corpus luteum is forming. There is evidence for the regulation of *ER β* in the ovary. Byers *et al.* (1997) showed that *ER β* mRNA was down-regulated after hCG injection in the rat. What was clear with the pattern of regulation *in vivo* and *in vitro* was that *ER β 1* and *ER β 2* appeared to be differentially regulated. What appeared consistent is the tendency for *ER β 1* to rise as the corpus luteum is fully formed or luteinised granulosa cells are cultured, whereas *ER β 2* tends to do the opposite. The reason for such apparent differential regulation is not clear.

It is obvious that granulosa-lutein cells of the corpus luteum express both *ER β 1* and *ER β 2*. It is not clear whether some cells have more *ER β 1* or whether this is a function of the detection system, but it seems that if a cell has *ER β 2* it also expresses *ER β 1*. It is also clear that any regulation of mRNA cannot be detected clearly at a protein level. This is in contrast to progesterone receptor, where down-regulation is evident in the late luteal phase *in vivo* and *in vitro* (Duncan *et al.* 2005a). It seems that *ER β s* are maintained and thus possibly active in the late luteal phase.

The reason for the apparent differential regulation for *ER β 1* and *ER β 2* is not clear. It should be noted that these findings represent mRNA rather than protein. However, *in vitro* both the acute hCG and oestradiol treatments tend to have the same effect on the mRNA expression of ERs. This is similar to the down-regulation of *ER β* mRNA by hCG in rat granulosa cells *in vitro* (Byers *et al.* 1997). The effect of chronic exposure to hCG is less marked, however, as there were no differences between late and rescued corpora lutea *in vivo* and in the corresponding treatments *in vitro*. The molecules involved in potential differential regulation remain to be determined.

We did not see any differential changes in the immunostaining of various cell types across the luteal phase. However, we did detect both *ER β 1* and *ER β 2* in endothelial cells. This is similar to what is seen in the human endometrium (Critchley *et al.* 2001) and in contrast to the localisation of progesterone receptors (Maybin & Duncan 2004). The corpus luteum is associated with intense angiogenesis (Reynolds *et al.* 2000, Wulff *et al.* 2001) followed by vascular regression. As oestradiol is produced by the granulosa-lutein cells (Devoto *et al.* 2002b) of the corpus luteum, with receptors on endothelial cells, it may have potential effects on endothelial cell function. It is not known whether oestradiol has any direct effects, but it is notable that molecules based on oestrogen have been shown to have anti-angiogenic properties (Klauber *et al.* 1997, Mooberry 2003, Tinley *et al.* 2003). Oestradiol may therefore have effects on the luteal vasculature.

The effects on granulosa-lutein cells are also not certain. In pilot experiments, we were able to see several effects of the pharmacological concentrations of oestradiol in the cultures of luteinised granulosa cells, but here we investigated the effects of physiological

concentrations (Andersen & Hornnes 1994). One major role of these cells is steroidogenesis, but we could not detect acute effects of oestradiol on the major regulators of luteal steroidogenesis *LHCGR* or *STAR*.

Both VEGF and CTGF are major regulators of tissue and vascular remodelling in the corpus luteum (Duncan *et al.* 2005b, Fraser & Duncan 2005), but oestradiol had no effect on their mRNA expression over 24 h. This was surprising as it has been demonstrated that VEGF has a functional oestrogen response element in its promoter (Hyder *et al.* 2000) and oestradiol has been shown to up-regulate *Ctgf* mRNA in rat granulosa cells (Harlow *et al.* 2007). Others have demonstrated a very rapid increase in VEGF expression after stimulating isolated human endometrial cells (Shifren *et al.* 1996) or human MCF-7 breast carcinoma cells (Ruohola *et al.* 1999) as rapidly as 1 h. It may be that such rapid effects do occur or that in these cells the major hormonal regulator is LH. The interaction of oestradiol and LH on VEGF expression in human luteinised granulosa cells remains to be fully investigated.

We also investigated the effect of oestradiol on two other molecules that seem to be important in human luteal function, namely activin (Myers *et al.* 2007a) and locally produced glucocorticoids (Myers *et al.* 2007b). Oestradiol regulated mRNA expression of *INHBA* and *HSD11B1*. The importance of this change is not clear, but it suggests the presence of functional ER signalling pathway in these cells.

How much of this is *ERβ1* and how much involves low levels of *ERα* expression is not clear. *ERβ2* is reported not to bind ligand (Matthews & Gustafsson 2003) and therefore does not activate the transcription of an oestrogen-sensitive reporter gene. It has a role in heterodimerisation, although with *ERα* more so than *ERβ1* (Ogawa *et al.* 1998). Whether the role for *ERβs* in the corpus luteum is to act directly or to function to remove potential classical oestrogen action by a dominant negative route remains unclear. Indeed, there is increasing evidence for the existence of membrane-bound ERs (Matthews & Gustafsson 2003) and effects of such molecules have not been considered here, but they may be important.

We have, however, shown the expression of *ERβ1* and *ERβ2* in the corpus luteum, suggested differential regulation and confirmed that luteinised granulosa cells are an additional tool to study ER signalling and differential regulation *in vitro*.

Materials and Methods

Collection of human ovarian tissue and human corpora lutea

Tissue collection was approved by the medical research ethics committee and all women gave informed consent. Normal ovarian tissue was collected from women with regular cycles

undergoing hysterectomy for benign conditions. Human corpora lutea ($n=18$) were enucleated at the time of surgery from women with regular menstrual cycles undergoing hysterectomy for benign conditions and dated on the basis of the urinary LH surge as described previously (Duncan *et al.* 1996, Duncan 2000). In this study, six corpora lutea were classified as early luteal (LH+1 to LH+5), six as mid-luteal (LH+6 to LH+10) and six as late luteal (LH+11 to LH+14). At operation, the corpus luteum was quartered to ensure that each quarter contained all cellular elements and was fixed in 10% (v/v) neutral buffered formalin for subsequent immunohistochemistry. In addition, archival corpora lutea that had been immediately frozen and stored at -70°C from previous studies (Duncan *et al.* 2005b, Fraser *et al.* 2005) were also available. Frozen tissue quarters for mRNA extraction were available from three early luteal, seven mid-luteal, six late luteal and five corpora lutea that had been 'rescued' (women were given daily doubling doses of exogenous hCG (Serono Laboratories, Welwyn Garden City, UK), starting at 125 IU, from LH+7 for 5–8 days until surgery) as described previously (Duncan *et al.* 1996, 1998b).

Isolation of human luteinised granulosa cells

The medical ethics committee separately approved the collection of cells from patients undergoing assisted conception. With patient consent, follicular fluid was collected from women undergoing transvaginal oocyte retrieval for *in vitro* fertilisation after ovarian stimulation using a standard procedure (Duncan *et al.* 2005a). Isolation of luteinised granulosa cells using Percoll density gradient centrifugation was carried out as described previously (Duncan *et al.* 2005b, Myers *et al.* 2007a).

HCG treatments in the primary cultures of luteinised granulosa cells

To investigate the acute effects of hCG and oestradiol, pooled luteinised granulosa cells (100 000 per well of 3–5 patients) were cultured in 24-well plates precoated with Matrigel (BD Biosciences, Bedford, MA, USA) in serum-free medium (supplemented DMEM/F12 Ham mixture), as described previously (Duncan *et al.* 2005b). Briefly, cells were refreshed with serum-free culture medium every 2 days after until day 6 when they were treated with either 100 ng/ml hCG (Serono Laboratories), or with 10^{-6} mol/l oestradiol in 0.1% (v/v) dimethyl sulphoxide (Sigma–Aldrich). Controls contained appropriate concentrations of the carrier solution.

To study the chronic effects of hCG, luteinised granulosa cells were cultured for 13 days as described previously (Duncan *et al.* 2005a). Briefly, cells were grown in 1 ng/ml hCG until day 7 when hCG was removed or increased to 100 ng/ml until day 13 of culture. Cells were analysed on day 2, 7 and 13 in the absence of hCG, and on day 13 with hCG. This regimen mimics the luteal phase and rescue (Duncan *et al.* 2005a).

In each experiment, three wells were pooled, in triplicate for each treatment for subsequent quantitative real-time PCR analysis. Each experiment was carried out at least three times to avoid biological bias.

Preparation of cDNA from luteinised granulosa cells cultures

Luteinised granulosa cell mRNA was extracted using RNeasy mini-spin columns after lysis by the addition of RNeasy lysis buffer (Qiagen). Lysates were frozen until processed as per manufacturers' protocols, then DNase treated with on-column DNaseI (Qiagen) and quantified using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). mRNA was then reverse transcribed into cDNA using random hexamers (Applied Biosystems, Foster City, CA, USA).

Quantitative analysis of gene expression by RT-PCR

Quantitative real-time PCR (QRT-PCR) was carried out on the ABI PRISM 7900 heat-cycler sequence detection system (Applied Biosystems) using specific primers and probes (Eurogentec, Southampton, UK) for each gene of interest (Table 1) and levels were related to a ribosomal 18s internal control (Applied Biosystems). All samples were performed in duplicate and a relative comparison was made to an appropriate control tissue cDNA.

Immunohistochemistry

Specific mouse monoclonal antibodies were used for the immunolocalisation of ER α (Clone 6F11, Vector Laboratories, Peterborough, UK), ER β 1 and ER β 2 (Clone PPG5/10 and PPG57/3 respectively; Serotec, Oxford, UK) using 5 μ m paraffin tissue sections of human ovary or human corpora lutea prepared on poly-L-lysine-coated microscopic slides. These sections were dewaxed, rehydrated, washed in PBS, subjected to antigen retrieval by boiling in a pressure cooker in 0.01 mol/l citric acid (pH 6.0) for 5 min and left to cool to room temperature. All sections were washed and placed in 3% (v/v) H₂O₂/methanol for 30 min, followed by an avidin and biotin block (Vector Laboratories) and a further block using normal goat serum (NGS, Diagnostics Scotland, Edinburgh, UK) diluted 1:5 in PBS containing 5% (w/v) BSA (NGS/PBS/BSA) for 1 h at room temperature. The sections were incubated overnight in primary antibody diluted 1:20 (ER α), 1:50 (ER β 2) or 1:100 (ER β 1) in NGS blocking solution at 4 °C.

All sections were then washed twice for 5 min in PBS plus 0.01% (v/v) Tween-20 (PBS-T; Sigma-Aldrich) before incubation with biotinylated goat anti-mouse secondary antibody (DAKO Corp., Cambridge, UK), diluted 1:500 in NGS blocking solution. Incubations lasted for 1 h and were followed by two washes in PBS-T for 5 min. Thereafter, the sections were incubated in avidin-biotin complex HRP (Vector Laboratories) for 1 h according to the manufacturer's instructions. All sections were washed in PBS-T (2 $\times$ 5 min) and bound antibodies visualised by incubation with liquid 3,3'-diaminobenzidine tetra-hydrochloride (DAKO). The sections were counterstained lightly with haematoxylin to enable cell identification. Negative controls for each antibody examined were performed identically to the above protocol with the primary antibody omitted or replaced with non-specific immunoglobulins. Images were captured using an Olympus Corp.

Table 1 List of all primer/probe sequences used for Taqman quantitative RT-PCR.

Gene	Fwd primer 5'-3'	Rev primer 5'-3'	Probe 5'-FAM-TAMRA-3'
HSD11B1 (NM_181755)	AGCTCTGCCCAAGAGAAGT	AGGATCTTCTGCATGGATTTC	TGACAGCTCACTCTGGACCACCTCTCTGA (Rae et al. 2004)
CTGF (NM_001901)	GTTTGGCCCAAGACCAACT	GGAAAGGCGCTCCACTCT	TGATTAGAGCCAACTGCTGCTCCAGA (Kirwan et al. 2005)
ER α (NM_000125)	TGATTGGTCTCGTCTGGCG	CATGCCCTCTACACATTTCCC	TGCTCCTAATTCTCTTGGACAGGAACC (Henderson et al. 2003)
ER β 1 (NM_001437)	CCTGGCTAACCTCCTGATGCT	CCACATTTTGACATTCATGTTG	AGAATGTTCCATGCCCTTGTTACTCGCA (Critchley et al. 2002)
ER β 2 (NM_001040275)	ATCCATGCCCTGGCTAAC	GAGTGTITGAGAGGCTTTTCTG	TCCTGATGCTCTCTGCCACGTC (Critchley et al. 2002)
INHBA (M13436)	GGACATCGGCTGGAAATGACT	GGCACTCACCTCGCAGTAG	ATCATTGCTCCCTGGCTATCATGCC (Casagrandi et al. 2003)
LHCGR (M63108)	CTGAATACTGATCCAGAACCAA	GCTCAAGTATTTAATCCGGGAAGA	ATCTGAGATACATTGAGCCCGGAGCAT (Ji et al. 2002)
STAR (NM_000349)	TTCCTTTATGGGCTCAAGATG	GGAGACCTCTGAGATCTGCTT	CATCGCTGGCAGTACATGTGCAC (Oskarsson et al. 2006)
VEGF (BC065522)	GTGCCCACTGAGGAGTCCA	GTGCTGGCCTTGGTGAGGT	CATCACCATGCAGATTATGGGATCAA (Kirwan et al. 2005)

Provis microscope (Olympus Corp. Optical Co., London, UK) equipped with a Kodak DCS330 camera (Eastman Kodak Co.), stored on a HP computer and assembled using Photoshop 7.0.1 (Adobe Systems Inc).

Fluorescent immunohistochemistry

Slides for the co-localisation experiments of ER β 1 with ER β 2 were washed, subjected to antigen retrieval and blocked as described above. Mouse anti-ER β 1, diluted 1:20 in NGS blocking solution, was incubated on the sections overnight at 4 °C. The sections were then washed in PBS (2 $\times$ 5 min) and the slides were incubated for 30 min with biotinylated goat anti-mouse Fab secondary antibody (Abcam, Cambridge, UK), diluted 1:500 in NGS blocking solution. After three washes in PBS, the sections were incubated for 1 h with avidin Alexa Fluor 488 (Molecular Probes, Paisley, UK) diluted 1:200 in PBS to amplify the ER β 1 immunostaining with green fluorescence.

For co-localisation with ER β 2, the sections were washed twice in PBS and then re-blocked with NGS/PBS/BSA containing non-conjugated goat anti-mouse Fab secondary antibody (Abcam) diluted 1:200 for 1 h and then incubated overnight at 4 °C with mouse anti-ER β 2 diluted 1:200 in NGS blocking solution. The sections were washed twice in PBS and incubated for 30 min with goat anti-mouse Fab HRP conjugated secondary antibody (Abcam) diluted 1:500 in NGS blocking solution before being washed twice in PBS and incubated for 10 min with tyramide Cy3 (TSA plus cyanine 3 system; Perkin–Elmer Life Sciences, Boston, MA, USA) diluted 1:50 in the supplied buffer to amplify the ER β 2 immunostaining with red fluorescence.

Slides for the co-localisation of ER β 1/ER β 2 with CD31/PECAM-1 were washed, subjected to antigen retrieval and blocked as described above. Mouse anti-CD31/PECAM-1 (Clone JC70A, DAKO), diluted 1:100 in NGS blocking solution, was incubated on the sections overnight at 4 °C. The sections were then washed twice in PBS and the slides were incubated for 30 min with goat anti-mouse Fab HRP conjugated secondary antibody (Abcam), diluted 1:500 in NGS blocking solution. After two washes in PBS, the sections were incubated for 10 min with tyramide Cy3 (TSA Plus Cyanine 3 System; Perkin–Elmer Life Sciences) diluted 1:50 in the supplied buffer to amplify the CD31/PECAM-1 immunostaining with red fluorescence.

For co-localisation with ER β 1 or ER β 2, the sections were washed twice in PBS and then re-blocked for 1 h with NGS/PBS/BSA containing non-conjugated goat anti-mouse Fab secondary antibody (Abcam) diluted 1:200 and then incubated overnight at 4 °C with mouse anti-ER β 1 or anti-ER β 2, both diluted 1:20 in NGS blocking solution. Sections were washed twice in PBS and incubated with biotinylated goat anti-mouse Fab secondary antibody (Abcam) diluted 1:500 in NGS blocking solution for 30 min. After two washes in PBS, sections were incubated for 1 h with avidin Alexa Fluor 488 (Molecular Probes) diluted 1:200 in PBS to amplify the ER β 1 or ER β 2 immunostaining with green fluorescence.

Slides were washed twice in PBS and mounted in Permafluor (Beckman Coulter, High Wycombe, UK). Fluorescent images were captured using an LSM 510 Axiovert 100 M confocal laser

microscope (Carl Zeiss Ltd, Welwyn Garden City, UK). All images were compiled using Photoshop 7.0.1 (Adobe Systems Inc).

Statistical analysis

Statistical analyses used are highlighted in the figure legends. Parametric statistics were used if the data were normally distributed with appropriate s.d.s (GraphPad Prism version 4.0c for Macintosh; GraphPad Software, San Diego, CA, USA). If the data were not normally distributed non-parametric statistics were used. Groups were analysed by Kruskal–Wallis non-parametric test and Dunn's multiple comparison test. Differences were considered significant at $P < 0.05$ level. The intensity of immunostaining for ER in granulosa-lutein cells, theca-lutein cells and stroma cells was carried out by an observer blinded to tissue identity as described previously (Duncan *et al.* 2005a).

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