

## Differential Expression and Functional Characterization of Luteinizing Hormone Receptor Splice Variants in Human Luteal Cells: Implications for Luteolysis

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The human LH receptor (LHR) plays a key role in luteal function and the establishment of pregnancy through its interaction with the gonadotropins LH and human chorionic gonadotropin. We previously identified four splice variants of the LHR in human luteinized granulosa cells (LGCs) and corpora lutea (CL). Real-time quantitative PCR revealed that expression of the full-length LHR (LHRa) and the most truncated form (LHRd) changed significantly in CL harvested at different stages of the ovarian cycle ( $P < 0.01$ , ANOVA). LHRa expression was reduced in the late luteal CL ( $P < 0.05$ ). Conversely, an increase in LHRd expression was observed in the late luteal CL ( $P < 0.01$ ). Chronic manipulation of human chorionic gonadotropin in LGC primary cultures supported the *in vivo* findings. LHRd encodes a protein lacking the transmembrane and carboxyl terminal domains. COS-7 cells expressing LHRd were unable to produce cAMP in response to LH stimulation. COS-7 cells coexpressing LHRd and LHRa also failed to generate cAMP in response to LH, suggesting that this truncated form has a negative effect on the signaling of LHRa. Immunofluorescence staining of LGC and COS-7 cells implied that there is a reduction in cell surface expression of LHRa when LHRd is present. Overall, these results imply expression of LHR splice variants is regulated in the human CL. Furthermore, during functional luteolysis a truncated variant could modulate the cell surface expression and activity of full-length LHR. (*Endocrinology* 150: 2873–2881, 2009)

The LH receptor (LHR) is a member of the G protein-coupled receptor (GPCR) superfamily and consists of an extracellular domain, seven-transmembrane domains connected by alternating intracellular and extracellular loops, and an intracellular carboxyl (C) terminal tail (1). The LHR has a pivotal role in human corpus luteum (CL) function through its interaction with the gonadotropins LH and human chorionic gonadotropin (hCG). Luteal progesterone production is dependent on LHR stimulation. During luteolysis progesterone production initially falls despite continued expression of LH (2), LHR (3), and factors downstream of the progesterone synthetic pathway (4). However, during pregnancy hCG, acting through the LHR, rescues the CL from luteolysis and progesterone secretion is main-

tained. Although this is crucial for human reproduction, how the CL is regulated at a molecular level is not known.

The LHR may increasingly lose its function as luteolysis is approached. Loss of LHR function occurs in patients with Leydig cell hypoplasia who have mutations in exons 8 and 10 of the gene (5, 6). Furthermore, human LHR without exon 10 is responsive to hCG but not LH (7). This LHR isoform is the naturally produced receptor in the marmoset monkey (8) in which CG is the significant pituitary gonadotropin (9). If the LHR variant without exon 10 exists in the human ovary, this could explain how the CL is able to respond to hCG and not LH during pregnancy.

Transcripts encoding alternatively spliced variants of the LHR have been detected in the rat (10), pig (11), sheep (12),

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Abbreviations: CL, Corpus luteum/corpora lutea; ER, endoplasmic reticulum; FSHR, FSH receptor; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; hCG, human chorionic gonadotropin; IBMX, isobutyl-1-methylxanthine; LDL, low-density lipoprotein; LGC, luteinized granulosa cell; LHR, LH receptor; LHRa, full-length LHR; LHRd, most truncated form.

horse (13), cow (14), and human (15) CL. A transcript variant with exon 10 spliced out was detected in the sheep (12) and cow (16) CL, but we did not identify this transcript in the human ovary (17). However, we did find three alternative splice variants of the LHR in human CL and luteinized granulosa cells (LGCs). *LHRa* encoded the full-length receptor. *LHRb* was missing exon 9, whereas *LHRc* and *LHRd* were lacking the first 266 nucleotides of exon 11. Exon 9 was removed in *LHRd*, too (17) (Fig. 1A). Exon 9 encodes part of the leucine-rich motif and hinge regions of the extracellular domain. *LHRb* was unable to bind hCG or activate second messenger signaling indicating that exon 9 is important for these functions (18, 19). The deletion of part of exon 11 causes a frame shift in the reading frame and creates a truncated protein that is missing amino acids 317–699 of the full-length sequence. The insertion of a different 13-amino acid stretch of sequence creates a unique C terminus preceding a premature stop codon. The *LHRc* and *LHRd* proteins are therefore missing part of the hinge region, transmembrane domains, and C-terminal tail.

FSH and follicle size regulate alternative splicing of the cow *LHR* (20). Furthermore, *LHRb* formed receptor complexes with, as well as negatively regulated the expression and activity of, *LHRa* and FSH receptor (FSHR) (19, 21, 22). A rat *LHR* truncated variant, similar in structure to human *LHRd*, also controlled cell surface expression of *LHRa* (23).

We hypothesized that as the human CL ages, there is an increase in the production of alternatively spliced *LHRs* that inhibit LH signaling. We studied the expression and regulation of *LHR* splice variants in human CL across the luteal phase and in LGCs. Additionally, we examined the functional significance of the most abundant splice variant, *LHRd*.

## Materials and Methods

### Collection of human CL

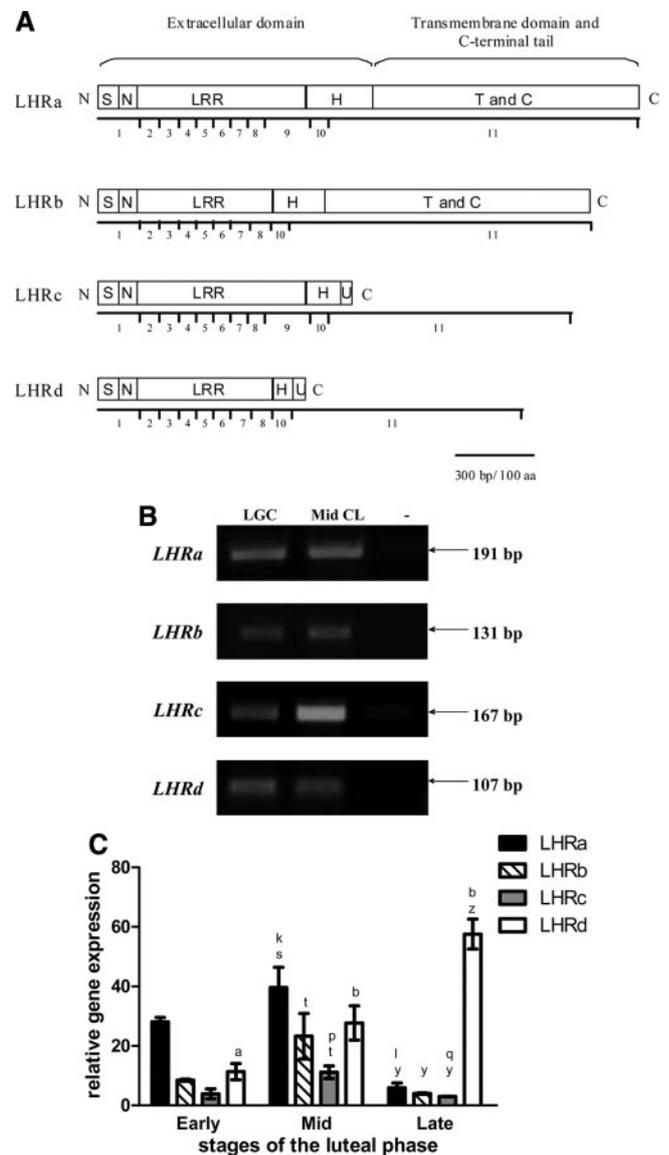
Tissue collection was approved by the local medical research ethics committee. With patient consent, human CL ( $n = 13$ ) were enucleated at the time of surgery from women with regular menstrual cycles undergoing hysterectomy for benign conditions and dated on the basis of the urinary LH surge (24). In this study, four CL were classified as early luteal (LH+1 to LH+5), four as midluteal (LH+6 to LH+10), and five as late luteal (LH+11 to LH+14).

### Isolation of primary cells

The medical ethics committee separately approved the collection of cells. With patient consent, follicular fluid was collected from women undergoing transvaginal oocyte retrieval for *in vitro* fertilization (25). LGCs were isolated using Percoll density gradient centrifugation (26, 27).

### Cell culture

All cells were maintained at 37°C, 5% CO<sub>2</sub>. Pooled LGCs (1–1.5 × 10<sup>5</sup> cells/well of three to five patients) were cultured in plates precoated with matrigel (BD Biosciences, Bedford MA) in serum-free medium (insulin transferrin-sodium selenite supplemented DMEM/F12 Ham mixture; Life Technologies, Inc., Gaithersburg, MD). After 6–8 d, cells were treated with recombinant hCG (Serono Laboratories, Welwyn Garden City, UK; 100 ng/ml) and low-density lipoprotein (LDL; Sigma-Aldrich



**FIG. 1.** A, Domain structure of LHR isoforms. The *LHRa* transcript contains all 11 exons and encodes the full-length receptor. The *LHRb* transcript is missing exon 9 and therefore encodes a protein lacking part of the LRR and H regions. The *LHRc* and *LHRd* transcripts are missing the first 266 nucleotides of exon 11, which causes a frame shift and premature stop codon. These transcripts are therefore missing the rest of exon 11, which encodes the T and C regions. The *LHRd* transcript is also lacking exon 9. S, Signal peptide; N, N-terminal cysteine-rich region; LRR, leucine-rich motif region; H, hinge region; T and C, transmembrane and C-terminal tail; U, unique C-terminal tail. B, Expression of *LHR* splice variants. RNA extracted from cultured LGCs and a midluteal phase CL (mid CL) was used as a template for RT-PCR using primers specific for each splice variant. All splice variants were expressed in LGCs and mid CL and primers produced a single band suitable for real-time quantitative PCR. C, Expression of individual *LHR* splice variants in staged human corpora lutea using real-time quantitative PCR. *LHRa* expression was significantly reduced in late luteal CLs compared with the midluteal phase ( $P < 0.01$ , Tukey). A similar nonsignificant trend was seen for *LHRb* expression in these samples. *LHRc* expression was also significantly lower in the late compared with the midluteal phase ( $P < 0.05$ , Tukey). In contrast, a significant increase in *LHRd* expression was observed in the late luteal CLs from the early and midluteal phases ( $P < 0.01$  and  $P < 0.05$ , respectively, Tukey). The proportion of LHR variants during each stage of the luteal phase also changed significantly ( $P < 0.001$ , two-way ANOVA). In the midluteal phase, there was a higher proportion of *LHRa* compared with *LHRb* and *LHRc* ( $P < 0.05$  and  $P < 0.001$ , respectively, Bonferroni). In the late-luteal phase, there was a higher proportion of *LHRd* compared with the other LHR variants ( $P < 0.001$ , Bonferroni). Values are the mean  $\pm$  SEM. Different consecutive letters represent statistical differences.

Corp., Gillingham, UK; 50 mg/liter); recombinant LH (Serono Laboratories; 10 ng/ml) and 50 mg/liter LDL; and recombinant human activin-A (R&D Systems, Inc., Abingdon, UK; 25 ng/ml) or cortisol (Sigma-Aldrich; 100 nM) for 24 h. Previous studies had determined that these doses of activin A and cortisol had a maximal effect on LGCs (28, 29). For each treatment controls contained an equivalent volume of the carrier solution and were conducted in parallel.

To mimic the luteal phase, LGCs were plated as described above and maintained for 12 d (25). Cells were stimulated with 1 ng/ml hCG and 50 mg/liter LDL from d 2 of culture, and this treatment was repeated every second day until d 7. The remaining cells were given either 50 mg/liter LDL or 100 ng/ml hCG and 50 mg/liter LDL for an additional 6 d. Cells were collected at d 3 and 7 along with d 13 in the absence or presence of hCG. Media were collected at the above time points and measured for progesterone levels using an in-house RIA (25).

COS-7 cells were routinely maintained in DMEM (Life Technologies) supplemented with 10% fetal calf serum, penicillin/streptomycin, and 2 mM glutamine (complete DMEM).

### Expression analysis

RNA was extracted from cells and tissue using the RNeasy Mini kit (QIAGEN Ltd., Crawley, UK) and treated with deoxyribonuclease I (QIAGEN). RNA was used as a template for cDNA synthesis using Taqman reverse transcriptase reagents (Applied Biosystems, Warrington, UK). Primers specific for the *LHR* variants were designed based on previous studies (17). PCR was performed on an Eppendorf Mastercycler gradient authorized thermocycler (PerkinElmer, Inc., Waltham, MA) using GoTaq Flexi DNA polymerase (Promega Ltd., Southampton, UK). The PCR thermocycle consisted of an initial denaturation of 5 min at 95 °C followed by 35 cycles of 95 °C for 30 sec, annealing temperature for 30 sec, 72 °C for 30 sec, and a final extension of 10 min at 72 °C. PCR products were purified using QIAquick gel extraction kit (QIAGEN) and verified by sequencing. Primers specific for the *LHR* variants were designed based on previous studies (17) (see Table 1).

### Real-time quantitative PCR

A standard curve was generated with serial dilutions of human CL cDNA. Real-time PCR amplification was then performed in duplicate 10- $\mu$ l reactions using Platinum SYBR Green quantitative PCR SuperMIX-UDG (Invitrogen) and the ABI 7900HT fast real-time PCR system instrument (Applied Biosystems) (26). Each sample was normalized on the basis of its glucose-6-phosphate dehydrogenase content by dividing the amount of target gene by the amount of housekeeping gene.

### Generation of plasmid constructs

LHRd was amplified by PCR from human CL cDNA using the Expand Long Template PCR system (Roche Diagnostics Ltd., Burgess Hill, UK) and cloned into the pGEM-T Easy vector (Promega). The pSG5-human wild-type *LHRa* construct was a gift from T. Hamalainen (Uni-

versity of Turku, Turku, Finland). These fragments were subcloned into the *KpnI* and *ApaI* sites of the pDsRed-Monomer-C1 (CLONTECH Laboratories Inc., Saint-Germain-en-Laye, France) and pEGFP-C1 (CLONTECH) vectors. They were also subcloned into the *KpnI* and *EcoRI* sites of the pCDNA3.1/mycHisA vector (Invitrogen). All these constructs were verified by sequencing.

### cAMP assay

COS-7 cells were transiently transfected with 15  $\mu$ g of myc/His-LHRa, myc/His-LHRd, myc/His-LHRa, and the pCDNA3.1mycHisA empty vector or both myc/His-LHRa and myc/His-LHRd by electroporation using a Bio-Rad gene pulser (Bio-Rad Laboratories, Hercules, CA) ( $1.25 \times 10^6$  transfected cells/well) (30). After 48 h, complete DMEM was removed and cells were maintained in serum-free DMEM for 24 h.

Cells were incubated with serum-free DMEM containing 222  $\mu$ g/ml isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich), a phosphodiesterase inhibitor, for 30 min, allowing the accumulation of intracellular cAMP. Next the cells were stimulated with recombinant LH (6.25 IU/ml- $6.25 \times 10^{-5}$  IU/ml) and IBMX in serum-free DMEM. Each treatment was carried out in duplicate. After 30 min, media were aspirated and cAMP concentrations in pooled cell lysates were quantified using a competitive enzyme immunoassay kit (R&D Systems). OD was measured using a Multiskan EX microplate photometer (Thermo Fisher Scientific Inc., Basingstoke, UK) at 450 nm. A standard curve was generated and sample concentrations were calculated using AssayZap computer software (Biosoft, Cambridge, UK).

### Confocal laser microscopy

COS-7 and LGCs were transiently transfected with pEGFP-C1-LHRa, pEGFP-C1-LHRd, pEGFP-C1-LHRa, and pDsRed-Monomer-C1-empty or pEGFP-C1-LHRa and pDsRed-Monomer-C1-LHRd. COS-7 cells were transfected by electroporation as described above, seeded in poly-D-lysine-coated glass-bottom culture dishes (MalTek, Ashland, MA) ( $1.9 \times 10^6$  cells) and grown in complete DMEM. LGCs were isolated from follicular fluid as described above, transferred to culture dishes ( $4 \times 10^5$  cells/well), and cultured in DMEM/F12 Ham supplemented with 10% fetal bovine serum 37 °C and 5% CO<sub>2</sub> for 24 h. Cells were then transfected with 1  $\mu$ g of the indicated constructs using FuGENE 6 (Roche). After 24–48 h, COS-7 and LGCs were washed twice with PBS, fixed in 4% neutral buffered formaldehyde for 20 min, and stored in PBS. Images were captured using a LSM510 Axiovert 100M confocal laser microscope (Carl Zeiss Ltd., Welwyn Garden City, UK).

### Statistical analysis

Parametric statistics were used as the data for all experiments were normally distributed. A paired *t* test was used when treatment and control samples were analyzed. A one-way ANOVA was conducted when more than two groups were compared and the data were categorized in one way. When group means were significantly different by one-way

**TABLE 1.** Primers used in the expression analysis of candidate genes

Gene	Primer locations (exons)	Primer sequence (5'–3')	MgCl <sub>2</sub> (mM)	Annealing temp (°C)	Product size (bp)
<i>LHRa</i>	(F) 9	ATTTGTCAATCTCCTGGAGGC	1.5	55	191
	(R) 11	CACTCAGTTCACCTCAGCA			
<i>LHRb</i>	(F) 8 and 10	AGGGCCGAAAACCTTACAGAA	1.5	55	131
	(R) 11	CACTCAGTTCACCTCAGCA			
<i>LHRc</i>	(F) 9	ATTTGTCAATCTCCTGGAGGC	1.5	55	167
	(R) 10 and 11	CCCCATGCAAAAAGTGTTTGG			
<i>LHRd</i>	(F) 8 and 10	AGGGCCGAAAACCTTACAGAA	1.5	54	107
	(R) 10 and 11	CCCCATGCAAAAAGTGTTTGG			
<i>G6PDH</i>	(F) 1	CGGAAACGGTCGTACTTTC	1.5	57	153
	(R) 2	CCGACTGATGGAAGGCATC			

Shown are each of the genes investigated, the exons to which primers were specific, primer sequences, and specific conditions used to amplify each product. The same primers were used for real-time quantitative PCR. F, Forward primer; R, reverse primer; G6PDH, glucose-6-phosphate dehydrogenase.

ANOVA ( $P < 0.05$ ), pairwise comparisons were performed using Tukey's multiple comparison test. A two-way ANOVA, followed by a Bonferroni posttest, was used when two or more data sets were compared for different factors. All statistical tests are highlighted in the figure legends.

## Results

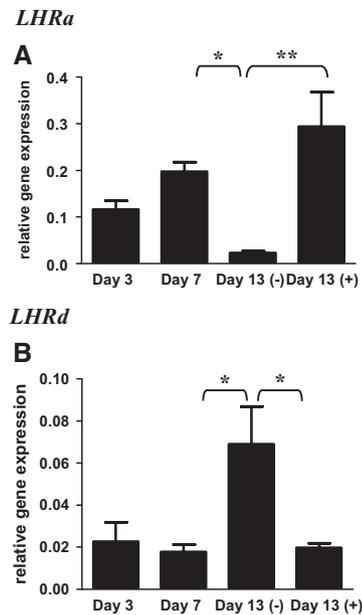
### Expression of truncated LHRs increased with CL age

Expression of *LHR* transcript variants was examined across the luteal phase in well characterized CL samples (Fig. 1, B and C). Real-time quantitative PCR revealed that expression of *LHRa* and *LHRd* changed across the luteal phase ( $P < 0.01$ , one-way ANOVA). *LHRa* expression was reduced by 7-fold in late-luteal CLs compared with the midluteal ( $P < 0.01$ ). In contrast, a 2- to 5-fold increase in *LHRd* expression was observed in the late-luteal CLs from the early and midluteal ( $P < 0.01$  and  $P < 0.05$ , respectively). *LHRc* expression also changed during the luteal phase and showed a similar trend to *LHRa* ( $P < 0.05$ ). A 4-fold decrease in *LHRc* expression was observed in the late-luteal CL compared with the midluteal phase ( $P < 0.05$ ).

The proportion of LHR variants during each stage of the luteal phase changed ( $P < 0.001$ , two-way ANOVA). In the midluteal phase, there was a higher proportion of *LHRa* compared with *LHRb* and *LHRc* ( $P < 0.05$  and  $P < 0.001$ , respectively, Bonferroni). In the late-luteal phase, there was increased expression of *LHRd* compared with the other *LHR* variants ( $P < 0.001$ , Bonferroni). Therefore, as the CL approaches luteolysis, there is an increase in the production of transcripts encoding truncated LHRs. Because *LHRa* and *LHRd* showed the most significant changes over the luteal phase, these variants were investigated further.

### Chronic manipulation of hCG increases *LHRa* and decreases *LHRd* in cultured luteinized granulosa cells

The CL contains many different cell types that may change in proportion across the luteal phase. To investigate the effects of time and hCG on *LHR* transcript variants, primary cultures of LGC were studied using an *in vitro* model designed to mimic the stages of the luteal phase (Fig. 2). After 13 d in the presence of increased hCG, progesterone concentrations were higher ( $1131 \pm 44.27$  ng/ml) than at d 3 or 7 of culture ( $P < 0.001$  and  $P < 0.01$ ). Conversely, progesterone concentration was lower at d 13 in the absence of hCG ( $119 \pm 10.12$  ng/ml) compared with d 7 and 13 with the gonadotropin ( $P < 0.001$ ). Using real-time quantitative PCR, *LHRa* and *LHRd* expression changed under these conditions ( $P < 0.01$  and  $P < 0.05$ ). Expression of *LHRa* was 9-fold lower when gonadotropin treatment was withdrawn ( $P < 0.05$ ) but increased by 12-fold under the presence of hCG ( $P < 0.01$ ). In contrast, *LHRd* expression was 4-fold higher when hCG was withdrawn ( $P < 0.05$ ) and 3.5-fold reduced in cultures with gonadotropin treatment ( $P < 0.05$ ), supporting the findings seen in the dated CL samples and suggesting hormonal regulation of LHR splicing.



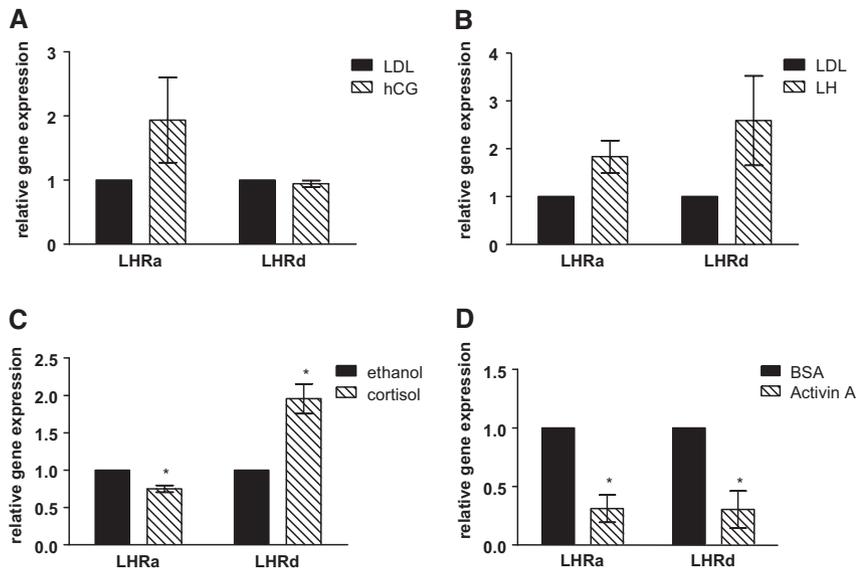
**FIG. 2.** *LHRa* and *LHRd* expression was regulated in prolonged cultures of LGCs designed to mimic the luteal phase. Cells were given low-dose hCG stimulation for 1 wk and RNA was extracted at d 3 and 7. At d 7 cells were then cultured for a further 6 d in the complete absence (–), or with a maximal dose (+), of hCG and RNA was extracted from these cells at d 13. A, Withdrawal of hCG led to reduction in *LHRa* expression ( $P < 0.05$ , Tukey); however, mRNA levels were restored in cultures given high doses of the hormone ( $P < 0.01$ , Tukey). B, Conversely, *LHRd* expression was maximal when hCG was withdrawn ( $P < 0.05$ , Tukey) and reduced in cultures with treatment ( $P < 0.05$ , Tukey). Values are the mean  $\pm$  SEM of three independent experiments. The asterisks correspond to  $P$  values (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

### Expression of *LHRa* and *LHRd* is acutely regulated by activin-A and cortisol in primary cell cultures

To determine whether the expression of *LHRa* and *LHRd* could be acutely regulated by hCG, its effect on primary cultures of LGCs was examined (Fig. 3A). Treatment with hCG for 24 h did not affect *LHRa* and *LHRd* expression. Likewise, the mRNA levels of *LHRa* and *LHRd* remained unchanged after LH treatment for 24 h (Fig. 3B). We also investigated whether cortisol could acutely modulate the expression of *LHRa* and *LHRd*. Cortisol treatment for 24 h diminished expression of *LHRa* by 1.4-fold and induced *LHRd* expression by 2-fold in LGCs ( $P < 0.05$ ) (Fig. 3C). Additionally, we assessed whether activin-A could differentially regulate expression of *LHRa* and *LHRd* in LGCs (Fig. 3D). The expression of both transcript variants was reduced by 3-fold in activin-A-treated cells ( $P < 0.05$ ).

### *LHRd* modulates the signal transduction by *LHRa*

Because these findings imply an inverse correlation in *LHRa* and *LHRd* expression during the CL life span, the function of the proteins they encode was investigated. *LHRd* is missing the transmembrane domains and C-terminal tail suggesting it is unable to activate downstream signaling pathways such as cAMP production. To confirm this hypothesis, COS-7 cells were transiently transfected with either myc/His-*LHRa* or myc/His-*LHRd*. The dose-response curves for *LHRa* and *LHRd* were different from each other ( $P < 0.001$ , two-way ANOVA). Treatment with LH caused a dose dependent increase in cAMP production in cells expressing *LHRa*. In contrast, a complete ab-



**FIG. 3.** Cortisol- and activin-A-regulated *LHRa* and *LHRd* expression in LGCs. The mean expression of control-treated cells was normalized to 1. Values are the mean  $\pm$  SEM of three independent experiments. A, Culturing LGCs with hCG (100 ng/ml) had no significant effect on *LHRa* and *LHRd* expression. B, The addition of LH (10 ng/ml) to cultures of LGCs for 24 h had a nonsignificant effect on *LHRa* and *LHRd* expression. C, Cortisol-treated LGCs had reduced *LHRa* and increased *LHRd* expression ( $P < 0.05$ , paired *t* test). D, Activin-A treatment caused a reduction in expression of both *LHRa* and *LHRd* ( $P < 0.05$ , paired *t* test). The asterisks correspond to *P* values (\*,  $P < 0.05$ ).

sense of signal transduction was seen in cells transfected with *LHRd* (Fig. 4A).

To examine whether *LHRd* may affect the signaling of *LHRa*, cAMP production was measured in COS-7 cells transiently co-transfected with myc/His-*LHRa* and either myc/His-*LHRd* or the pCDNA3.1mycHisA empty vector. The cAMP response of cells coexpressing *LHRa* and *LHRd* to recombinant LH was different from cells transfected with *LHRa* and the empty vector ( $P < 0.001$ , two-way ANOVA). After stimulation with recombinant LH, cells coexpressing *LHRd* and *LHRa* had undetectable cAMP levels. Conversely, cells transiently transfected with *LHRa* and the empty vector responded to LH in a dose-dependent manner (Fig. 4B).

### **LHRd effects the localization of LHRa**

The absence of transmembrane and C-terminal domains suggested that *LHRd* would not be localized to the plasma membrane. Immunofluorescence staining of COS-7 cells transiently transfected with green fluorescent protein (GFP)-*LHRd* confirmed that the variant was localized intracellularly. GFP-*LHRa*, on the other hand, was expressed at the cell surface in COS-7 cells (Fig. 5A). This finding was verified in LGCs exogenously expressing GFP-*LHRa* or GFP-*LHRd* constructs (Fig. 5B).

To investigate whether *LHRd* causes misrouting of *LHRa*, COS-7 cells were transiently transfected with GFP-*LHRa* and either RFP-*LHRd* or the pDsRed-Monomer-C1-empty vector. In COS-7 cells transfected with *LHRa* alone, *LHRa* was apparent in the plasma membrane and a diffuse perinuclear network. Conversely, in COS-7 cells coexpressing both isoforms, *LHRa* and *LHRd* showed similar staining inside the cell (Fig. 5C). Comparable results were seen in LGCs cotransfected with GFP-*LHRa* and either RFP-*LHRd* or the pDsRed-Monomer-C1-empty vector (Fig. 5D).

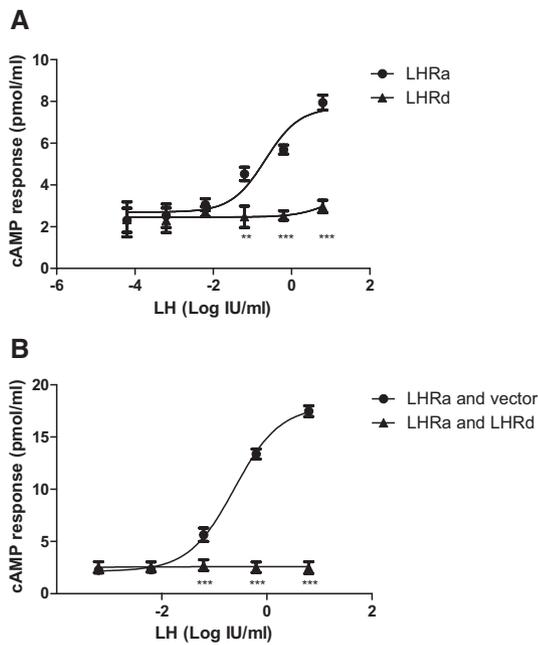
## **Discussion**

Previously we detected expression of the full-length *LHR* and three other splice variants in human CL (17). We were unable, however, to assess whether the relative expression of each transcript variant changed across the luteal phase. In this study we designed primers specific for each transcript variant and used real-time quantitative PCR to quantify the *LHR* transcripts. There was significantly increased *LHRd* and reduced *LHRa* expression in the late-luteal CL samples. An *in vitro* model, in which the luteal phase was mimicked in LGCs, mirrored these findings. Reduction of LHR signaling using hCG withdrawal increased *LHRd* expression, whereas increasing hCG concentrations maintained full-length *LHRa* expression.

In humans it is not known how luteolysis is regulated at the molecular level. It seems that the *LHR* expression in human corpora lutea is lower during luteolysis (18). However, in the late-luteal phase, when progesterone secretion is falling *LHR* expression is maintained (3). Because LH and the steroidogenic machinery (4) are retained, changes in functional LHR are implicated in the demise of the CL (31). Previous experiments (3, 21) on human luteal *LHR* expression used Northern blotting, and it was not clear whether the probe used was detecting all or just some of the *LHR* variants. Although total *LHR* expression might be maintained in the functional human CL, it appears, from our data, that expression of the full-length receptor is reduced in the late luteal phase, whereas there is increased expression of truncated LHR variants.

Minegishi *et al.* (21) also recently used real-time quantitative PCR to compare expression of *LHR* transcripts with and without exon 9 across the luteal phase. There was increased expression of *LHR* transcripts without exon 9 compared with variants with exon 9 as the luteal phase progressed, supporting our data. Because their primers theoretically detected both *LHRb* and *LHRd*, their results are difficult to fully relate to our findings with regard to *LHRb* expression in which we did not see an increase in the late luteal phase. However, expression of truncated *LHR* splice variants seems to increase at the expense of the full-length receptor as the CL matures.

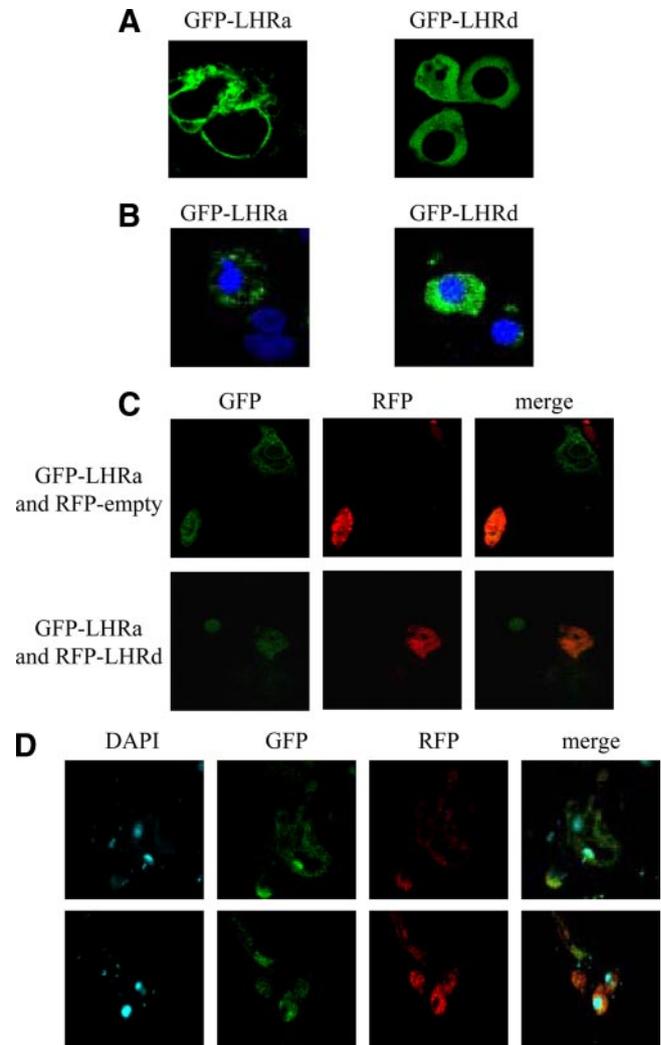
Our studies measured mRNA expression, so it is not known whether these *LHR* variants were translated into proteins in the CL or whether there was posttranslational modification over the luteal phase. Despite numerous attempts, we have not been able to identify a sensitive and specific antibody to the human LHR to test this fully by immunoblotting. However, in the sheep ovary a LHR splice variant with partial deletion of exon 11, encoding a protein with no transmembrane or intracellular domains, was translated *in vivo* (32). This is supported by the observation that our *LHRd* constructs were translated in LGC and COS-7 cells.



**FIG. 4.** LH stimulation of cAMP production by COS-7 cells expressing variants of the LHR. COS-7 cells were transiently transfected with myc/His tagged LHRa, myc/His tagged LHRd, myc/His tagged LHRa and the pCDNA3.1mycHisA empty vector, or both myc/His tagged LHRa and myc/His tagged LHRd. Cells were then stimulated with serial dilutions of recombinant LH and 222  $\mu$ g/ml IBMX for 30 min. The cAMP parameter assay (Roche) was conducted according to the manufacturers' instructions. Values are the mean  $\pm$  SD of three independent experiments. A, COS-7 cells expressing LHRa responded to LH, whereas an absence of signal transduction was seen in cells transfected with LHRd. The dose-response curves for LHRa and LHRd were significantly different ( $P < 0.001$ , two-way ANOVA). B, The presence of LHRd ablated the LHRa cAMP response to LH in transiently transfected COS-7 cells. The cAMP accumulation in the COS-7 cells transfected with LHRa and LHRd was at undetectable levels. The dose response curves for cells transfected with LHRa and either LHRd or an empty vector were significantly different ( $P < 0.001$ , two-way ANOVA). The asterisks denote statistically significant differences between the two curves by Bonferroni and correspond to  $P$  values (\*\*,  $P < 0.01$ ; or \*\*\*,  $P < 0.001$ ).

In prolonged cultures of LGCs treated with hCG, with increased progesterone concentrations, imitating the effects of early pregnancy, there was significantly increased expression of *LHRa* and diminished mRNA levels of *LHRd*. This suggests that *LHR* transcripts are regulated in luteal cells and could be influenced by the cellular environment. Regulation by hCG could be influencing *LHR* splicing, or it may be controlling gene transcription and/or mRNA degradation. Recent reports in the rat ovary have suggested that hCG treatment induces the expression of mevalonate kinase, which can then bind to *LHR* mRNA and accelerate its degradation (33). Whether mevalonate kinase has a similar role in humans and has different affinities to all the *LHR* transcripts requires investigation. It is difficult to know how hCG affects the expression pattern of *LHR* variants. We believe that progesterone could be mediating the effect because we have shown that blockage of progesterone is toxic to these cells (25, 29). Estradiol can enhance FSH-induced *LHR* mRNA in cultured rat granulosa cells (34). We did not include a substrate for estradiol in our medium, so we cannot exclude the possibility that estrogen could be involved in the regulation of *LHR* transcripts.

It is possible that one of the roles of hCG during maternal recognition of pregnancy is to maintain more full-length *LHRa*



**FIG. 5.** Localization of LHR variants in COS-7 cells and LGC. A, LHRa and LHRd were subcloned into the pEGFP-C1 vector (CLONTECH) and then transiently transfected into COS-7 cells. Whereas LHRa was localized to plasma membrane, LHRd showed a cytoplasmic distribution. B, The findings were confirmed in LGCs transiently transfected with either GFP-LHRa or GFP-LHRd. The cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). C, LHRd was also subcloned into the pDsRed-Monomer-C1 vector (CLONTECH). COS-7 cells were transiently transfected with GFP-tagged LHRa and either red fluorescent protein (RFP)-tagged LHRd or the pDsRed-Monomer-C1 empty vector. When the RFP empty vector was present, LHRa was localized mainly to the plasma membrane and perinuclear network. In cells expressing LHRa and LHRd, both proteins colocalized inside the cell. D, A similar result was observed in LGCs exogenously expressing GFP-LHRa and either RFP-LHRd (bottom panel) or pDsRed-Monomer-C1-empty (top panel). The cell nuclei were stained with DAPI. Results are representative of three independent experiments.

expression as well as global *LHR* expression (3). Ideally cAMP and ligand binding experiments would have been performed on the prolonged cultures of LGC when hCG was withdrawn to investigate whether there was a decrease in cell surface LHRs during the *in vitro* luteal phase. However, these cells are terminally differentiated, do not divide, collected in small amounts, and increasingly disappear from prolonged cultures (27). Therefore, we were not able to obtain the large number of cells required for such experiments. Whereas past studies have measured cAMP production in LGCs, measurements were made after culturing for 48 h, not 13 d (35). Furthermore, it would have been interesting to extend the culture course to see the effect of hCG

on the expression of LHRa and LHRd in a long-term culture system. However, primary LGCs have a 14-d life span and only survive under the influence of maximal hCG doses. Therefore, we would be unable to conduct controls for this experiment.

Although short-term exposure to LH or hCG did not significantly alter *LHR* splicing, other local factors could influence *LHR* expression. We have shown that activin-A has luteolytic properties in human luteal cells and therefore could be a possible regulator of LHR expression (28). However activin-A reduced expression of all four LHR splice variants to a similar level. In the rat activin-A facilitates granulosa cell function (36) and enhances p450 aromatase activity, a hallmark of gonadotropin action, in rat (37) and bovine granulosa cells (38) as well as immature and preovulatory marmoset follicles (39). Conversely, activin-A seems to have an opposing role in human lutein cells in which it hindered p450 aromatase activity and expression along with estradiol production (40, 41). Additionally, activin decreased basal and hCG-induced progesterone production in human granulosa lutein cells (42) and cultured macaque luteal cells (43). Overall, it seems that activin-A can suppress *LHR* expression, and this may have a role during luteolysis. However, activin-A is not responsible for the differential expression of LHR transcript variants.

The steroid cortisol did appear to influence *LHR* expression in LGC cultures. This glucocorticoid significantly diminished *LHRa* and induced *LHRd* expression in cultured LGCs. Whereas this has not been reported in the human ovary before, the full-length *LHR* mRNA was reduced by cortisol in cultures of porcine granulosa cells (44). Furthermore, the number of LHRs in cultured bovine granulosa cells was significantly reduced by cortisol in a dose-dependent manner (45). Previous findings have also implicated other glucocorticoids in inhibition of testicular LHR content and steroidogenesis (46) along with LH-induced steroidogenic acute regulatory protein levels and progesterone production in cultured rat preovulatory follicles (47). The mechanism by which glucocorticoids exert this effect may involve influencing the synthesis of membrane phospholipids (48) or hampering intracellular cAMP (45). However, we have shown that local cortisol generation is associated with hCG action and luteal rescue rather than luteolysis (26, 29). Cortisol also seems to negatively regulate putative luteolytic factors (26). It is therefore not clear what the functional role of cortisol is with regards to LH action, and further research is needed.

Our *in vivo* and *in vitro* results implied that as the CL ages, there is increased production of the most truncated form of the receptor. This isoform, LHRd, lacks the transmembrane and C-terminal domains, suggesting this truncated receptor would be unable to activate second-messenger signaling. Due to the limitations of working with terminally differentiated primary cells, we used COS-7 cells to investigate the functional properties of LHRd. COS-7 cells have been used successfully in the past as a model for examining the biological significance of human naturally occurring LHR mutants (7) and splice variants (18). COS-7 cells expressing LHRd and stimulated with LH did not produce cAMP, confirming this prediction. Furthermore, COS-7 cells expressing LHRa and LHRd did not generate cAMP in response to LH. This indicated that LHRd may have a negative

effect on LHRa cell surface expression. Similar results have been seen in experiments with LHRb (19). This implies that the N-terminal and LRR1-7 regions of the extracellular domain could be involved in the phenomenon rather than the hinge region, transmembrane segments, loops, and C-terminal tail. Truncated forms of other GPCRs may also inhibit the signaling of their corresponding wild-type receptor (49, 50).

The rodent LHR can undergo desensitization from its ligand or uncoupling from second-messenger systems (51). During human luteolysis there is a decline in LH-induced progesterone synthesis despite continued LH secretion (38) and expression of other members of the steroidogenic pathway such as steroidogenic acute regulatory protein, P450 side-chain cleavage, and 3 $\beta$ -hydroxysteroid dehydrogenase (4). There also seems to be no ligand-induced down-regulation of LHR because previous studies have shown maintenance of mRNA levels and ligand binding throughout the menstrual cycle (3). In these studies total *LHR* mRNA and ligand binding were measured. This does not account for the possible negative effects truncated LHR isoforms may have on the full-length receptor's activity. Our results would imply that in humans a variant LHR could inhibit the cell surface expression of the full-length LHR, and this may have an important contribution to luteolysis.

Immunofluorescence staining, in LGC and COS-7 cells, revealed that LHRd is localized intracellularly. LHRb and a rat LHR truncated splice variant, with partial deletion of exon 11, reside mainly in the endoplasmic reticulum (ER) due to misfolding (19, 23). Furthermore, the rat variant was found to localize with the ER chaperones calnexin and calreticulin in a specialized juxtannuclear subcompartment of the ER before being targeted for the ER associated degradation pathway (23). These ER chaperones have also been shown to associate with immature forms of human LHR, FSHR, and TSH receptor along with misfolded LHR mutants (52). In LGC and COS-7 cells transfected with LHRd, LHRa seemed to be localized within the cell. However, we did not quantify the result, so we cannot exclude the possibility that there is still some cell surface expression of LHRa. Furthermore, the rat LHR splice variant, lacking transmembrane and C-terminal domains, colocalized with immature LHRs and redistributed them to the juxtannuclear ER subcompartment (23). Splice variants of other GPCRs appear to have a comparable effect and function as dominant negatives to mediate misrouting of their corresponding wild-type receptors to the ER (19, 23, 50, 53, 54). Moreover, coexpression of the rat LHR splice variant, missing transmembrane, and C-terminal domains, with the full-length LHR, resulted in a reduced number of LHRs at the cell surface (23).

Research in the bovine indicated that detergent solubilization of cells transfected with truncated LHR splice variants, with a structure akin to LHRd, have the same hCG binding affinity as the full-length LHR. No binding activity was observed in intact cells expressing these forms though (16). In addition, past findings have implied that LHRb, missing exon 9, is unable to bind hCG at the cell surface (19). Because LHRd is missing exon 9, as well as part of exon 11, it is likely that this variant is incapable of interacting with either ligand at the cell surface, although we did not test this hypothesis directly. Previous research demon-

strated  $^{125}\text{I}$ -LH binding activity in thin tissue sections and tissue homogenates at all stages of the luteal phase and during maternal recognition of pregnancy (3, 55). In both these studies, however, it is difficult to conclude whether there were changes in LHR numbers at the cell surface across the luteal phase.

It is possible that the truncated variant functions as a chaperone or degradation assistant, and this warrants further elucidation. Misrouting of the full-length receptor could be a result of dimerization with the variant. Past findings have shown that LHRb, missing exon 9, interacts with and reduces the expression of the full-length LHR as well as the FSHR (19, 22). In these studies the receptor complexes were transferred to the lysosome in which they were eventually degraded. Additionally, previous research indicated that the FSHR extracellular domain is able to dimerize in the absence of the C-terminal half of the protein (56). Further studies are required to investigate whether LHRa and LHRd can heterodimerize. However our preliminary studies (data not shown), and research of Apaja *et al.* (23) using the rat LHR variant missing the transmembrane and C-terminal domains, failed to detect dimerization of the truncated LHR with the full-length receptor by coimmunoprecipitation. This suggests that a stable interaction between this particular LHR variant and LHRa is unlikely to take place. It is possible that cotransfection of LHRd with LHRa was having an indirect effect on the expression of the full-length receptor, possibly through exhausting transcriptional and/or translational machinery. Data on LHRb, however, indicated that whereas it reduces LHR and FSHR cell surface expression, it has no effect on the TSH receptor (19, 21, 22). We are currently dissecting the underlying mechanism by which LHRd reduces LHRa cell surface expression.

In conclusion, *in vivo*, as the CL approaches luteolysis, there is increased expression of *LHRd*, the most truncated form of the LHR. Furthermore, mimicking luteal rescue of pregnancy, by treating LGC cultures with hCG, increased *LHRa* and decreased *LHRd* expression. Whereas we have not confirmed whether this variant is translated into a protein in the human CL, *in vitro* expression studies in LGC and COS-7 cells suggest LHRd reduces cell surface expression of LHRa. Overall, our findings indicate that the expression of LHR transcript variants is regulated in the human CL, and during luteolysis a truncated splice variant negatively regulates the cell surface expression and activity of the full-length LHR.

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