Gonadotrophins modulate hormone secretion and steady-state mRNA levels for activin receptors (type I, IIA, IIB) and inhibin co-receptor (betaglycan) in granulosa and theca cells from chicken prehierarchical and preovulatory follicles

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Abstract

Ovarian follicle development is regulated through endocrine and local mechanisms. Increasing evidence indicates roles for transforming growth factor β superfamily members, including inhibins and activins. We recently identified divergent expression of mRNAs encoding activin receptors (ActR) and inhibin co-receptor betaglycan in chicken follicles at different stages of maturation. Here, we compare the actions of LH and FSH (0, 1, 10, 100 ng/ml) on levels of mRNA for ActRI, ActRIIA, ActRIIB and betaglycan in chicken granulosa and theca cells (GC and TC) from preovulatory (F1) and prehierarchical (6–8 mm) follicles. The expression of mRNAs for LH-R and FSH-R and production of inhibin A, oestradiol and progesterone were also quantified. FSH decreased ActRIIB and ActRI mRNA levels in 6–8 mm GC, whereas LH increased the mRNA levels. Both LH and FSH enhanced ActRIIA (5- and 8.5-fold) and betaglycan mRNA expression (2- and 3.5-fold) in 6–8 mm GC. In 6–8 mm TC, LH and FSH both increased the betaglycan mRNA level (7- and 3.5-fold respectively) but did not affect ActRI, ActRIIA and ActRIIB transcript levels. In F1 GC, both LH and FSH stimulated ActRI (2- and 2.4-fold), ActRIIB (3.2- and 2.7-fold) and betaglycan (7- and 4-fold) mRNA levels, while ActRIIA mRNA was unaffected. In F1 TC, LH and FSH reduced ActRIIA (35–50%) and increased (4.5- and 7.6-fold) betaglycan mRNA, but had no effect on ActRI and ActRIIB transcript levels. Results support the hypothesis that expression of ActR and betaglycan are differentially regulated by gonadotrophins during follicle maturation in the hen. This may represent an important mechanism for fine-tuning follicle responsiveness to local and systemic activins and inhibins.

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Introduction

The avian ovary provides a unique model for the study of folliculogenesis. The single functional left ovary typically contains approximately six (F1–F6) preovulatory follicles arranged in a strict size hierarchy with follicles committed to ovulate at intervals of 24–26 h. After ovulation of the largest (F1) follicle, the succeeding follicles each move up one place in the hierarchy and an additional follicle is recruited from the population of small yellow (6–8 mm diameter) prehierarchical follicles. Prehierarchical follicles undergo a higher rate of atresia than preovulatory follicles (Gilbert *et al.* 1983). Various ligands including inhibins (Inh), activins (Act), steroids (Bahr & Johnson 1984, Lovell *et al.* 1998) and receptors including activin receptors (ActR; Lovell *et al.* 2006), inhibin co-receptor (betaglycan, β gly; Sweeney & Johnson 2005, Lovell *et al.* 2006) and receptors for the

gonadotrophins luteinizing hormone and follicle-stimulating hormone (LH-R, FSH-R; Johnson *et al.* 1996, You *et al.* 1996) are also expressed to different extents by granulosa and theca cells (GC and TC) of prehierarchical and preovulatory follicles.

LH and FSH play key roles in regulating ovarian dynamics through stimulation of steroidogenesis, production of peptide hormones (including Inh and Act), cell proliferation (Bahr & Johnson 1984, Tilly *et al.* 1991, Johnson 1993, Lovell *et al.* 2002a) and apoptosis (Johnson *et al.* 1999) through specific LH and FSH receptors (Johnson *et al.* 1996, You *et al.* 1996). Inh and Act have been implicated as direct (paracrine/autocrine) and indirect (endocrine) regulators of ovarian development in birds (Davis *et al.* 2001, Johnson *et al.* 2004; review: Knight *et al.* 2005) and mammals (reviews: Knight & Glister 2003, Philips 2005).

The action of Act is elicited through a well-characterised signal transduction cascade (review Abe *et al.* 2004). Act can bind with high affinity to a type-II cell-surface receptor (ActRII) of which two have been cloned in avian (Ohuchi *et al.* 1992, Nohno *et al.* 1993, Stern *et al.* 1995) and mammalian species (Donaldson *et al.* 1992, Stern *et al.* 1995). Binding promotes the recruitment of activin type-I receptors (ActRI) to the complex which is then able to mediate ligand-dependent signalling by Smads (Knight & Glistler 2003, Abe *et al.* 2004). β gly (also known as transforming growth factor β (TGF β) III receptor) is a membrane-bound proteoglycan, which can bind inhibin and increase its affinity for ActRII (Lewis *et al.* 2000). This Inh/ β gly/ActRII complex is thought to prevent binding of Act to the ActRII and thereby block the recruitment of ActRI and activin-stimulated signal transduction (Phillips & Woodruff 2004).

During follicle development in the laying hen, changes have been observed in Inh/Act subunit mRNA expression (Davis & Johnson 1998, Knight *et al.* 2005), InhA, InhB, ActA and follistatin protein content (FS; Lovell *et al.* 1998, 2003) and expression of mRNAs for ActR (type I, IIA, IIB; Lovell *et al.* 2006) and β Gly (Sweeney & Johnson 2005, Lovell *et al.* 2006). ActRIIA mRNA expression has also been shown (by Northern blot) to change throughout follicle development in broiler breeder hens (Slappeg & Davis 2003). This suggests a functional involvement of the intraovarian Inh-Act system in follicle progression. Similarly, marked differences in steroidogenic capacity (Etches & Duke 1984, Kato *et al.* 1995) and mRNA expression of gonadotrophin receptors (LH-R, FSH-R; Johnson *et al.* 1996, You *et al.* 1996) have been recorded within both GCs and TCs during follicle development in birds.

The observation that treatment of hen preovulatory follicle GCs with ActA greatly enhanced gonadotrophin-induced InhA and progesterone (P4) release suggests a functional interaction between gonadotrophin-dependent and Act-dependent signalling pathways (Lovell *et al.* 2002a). In support of this, ActA was recently shown to enhance the expression of both FSH-R and LH-R in GC from preovulatory follicles (Johnson *et al.* 2006). There are other reports (Johnson *et al.* 2004, 2006, Woods & Johnson 2005) that ActA augments gonadotrophin receptor mRNA expression in granulosa cells from prehierarchical (small yolky) hen follicles.

With the above findings in mind, the primary aim of the present study was to test the hypothesis that pituitary gonadotrophins differentially modulate granulosa expression of ActRs and β gly mRNAs at two key stages of folliculogenesis: prehierarchical and preovulatory. In the absence of comparable studies on avian TC, we also conducted parallel experiments to determine the effects of gonadotrophins on TC from both prehierarchical and preovulatory follicles.

Materials and Methods

Experimental animals

Laying hens (Goldline) towards the end of the first year of lay, with a clutch average of at least five eggs, were caged individually and maintained as required by the United Kingdom Home Office Regulations. Hens were kept on a standard long-day photo-schedule of 16 h light:8 h darkness cycle, at an ambient temperature of 21–23 °C. Food and water were available *ad libitum*. Ovipositions were monitored using time-lapse recording technology and used to predict the time of ovulation. Hens ($n=5-6$ different hens per culture) were killed by cervical dislocation ~12 h after ovulation of a mid-sequence egg and the largest preovulatory follicle (F1) and all 6–8 mm prehierarchical follicles were removed and placed immediately in sterile Hank's Balanced Salt Solution (HBSS; Gibco-BRL).

Isolation of granulosa and theca cells

Granulosa and theca layers were separated under aseptic conditions (Gilbert *et al.* 1977). In brief, granulosa and thecal tissue derived from a given follicle position in different hens were combined (creating separate granulosa and theca follicle pools from the 6–8 mm and the F1 follicles). Pooled tissues were enzymatically dispersed as described by Lovell *et al.* (2002b) except that thecal tissue was dispersed for a further 15 min (total: 45 min). It should be noted that the theca tissue collected for dispersion consists of the theca interna and theca externa layers and therefore all the different cell types these layers may contain *in vivo*. The number of viable cells, estimated by trypan blue exclusion (Sigma UK Ltd), was invariably >90% for both the cell types. Isolated cells were diluted in incubation medium (medium 199 with 25 mM Hepes, 0.01% (v/v) L-glutamine and 1% (v/v) antibiotic solution) containing 2% (v/v) charcoal-stripped fetal calf serum (Sigma UK Ltd) and divided into aliquots in 24-well plates (Falcon 3047; Becton Dickinson Labware, Franklin Lakes, NJ, USA) at 5×10^5 viable cells per well. The cells were maintained for 24 h at 39 °C and the unattached cells were aspirated off and the adherent cells were washed three times with 1 ml serum-free incubation medium. All further incubations were done in serum-free conditions. Incubation buffer (1 ml) and test treatments were added to the appropriate wells. The cells were incubated for 2×24 h treatment periods with the media replenished with the appropriate test treatments after 24 h. At termination of the culture, the media were removed and stored at –20 °C and the cell monolayers were washed three times with PBS (Oxoid Ltd, Basingstoke, Hampshire, UK) before lysis in Tri-reagent (Sigma UK Ltd).

Treatments

Stock solutions of ovine (o)LH (NIDDK-oLH-25) and oFSH (NIDDK-oFSH-19-SIAPH) were prepared at 10 µg/ml in HBSS containing 0.3% (v/v) BSA (fraction V; Sigma UK Ltd). Before culture, the treatments were diluted in the above incubation medium, to a 40× treatment concentration and filter sterilised using a 0.2 µm Millipore filter (FlowPore D; ICN Biomedicals Ltd, Basingstoke, Hampshire, UK). Both gonadotrophins were tested at final concentrations of 1, 10 and 100 ng/ml.

RNA purification and cDNA synthesis

RNA was purified from cell monolayers according to the standard Tri-reagent protocol (Sigma UK Ltd). The final RNA pellet was resuspended in 100 µl nuclease-free water containing RNA Secure (Ambion, Huntingdon, Cambridgeshire, UK) and then treated with RNase-free DNase (15 min at 37 °C; RQ1, Promega) to remove potential genomic DNA contamination. The RNA preparation was re-purified using 15 volumes Tri-reagent. The resultant purified RNA was resuspended in 50 µl nuclease-free water containing RNA Secure.

RNA was quantified on a spectrophotometer (Gene-Quant, GE Healthcare UK Ltd, Amersham) and cDNA was synthesised using ImProm-II Reverse Transcriptase (Promega; used according to the Manufacturers instructions) with 1 µg RNA, 0.5 µg Random Hexamer Primers (MWG-Biotech, Covent Garden, London, UK), dNTPS (0.5 mM Final; Promega) and 0.5 µl RNase-inhibitor (40 U/µl; Ambion) per reaction. cDNA synthesis was terminated by heat-inactivation (15 min at 70 °C). cDNA samples were treated with 1 µl RNase cocktail (0.5 U/µl RNase A and 20 U/µl RNase T1; Ambion) and 0.5 µl RNase H (40 U/µl; Ambion), which specifically degrades the RNA in RNA:DNA hybrids. A 1 µl aliquot of cDNA was removed for estimation using a fluorometric assay (Oligreen ssDNA Quantification assay; Molecular Probes Inc., Paisley, Renfrewshire, UK).

Quantitative PCR

Duplicate Q-PCRs were carried out using 1 µl RT reaction product or 1 µl standard (from 200 to

1.56 amol/µl; standard oligonucleotides were custom-synthesised by Sigma-Genosys), in a volume of 25 µl containing 12.5 µl master mix with 1 µl ROX dye (Abgene, Epsom, Surrey, UK), 2 µl forward and reverse primers were each added (final concentration: 300–900 nM), 1 µl probe (final concentration: 100–200 nM) and 5.5 µl nuclease-free water. All forward and reverse primer working concentrations were 900 nM except the forward primer for GAPDH (300 nM). Probe working concentrations were 200 nM except for LH-R (150 nM), FSH-R (150 nM) and GAPDH (100 nM). The samples were processed for 40 cycles using an ABI Prism 7700 Sequence detector (Applied Biosystems, Warrington, Cheshire, UK) with the thermal cycler conditions; stage 1: 50 °C/2 min, stage 2: 95 °C/15 min, stage 3: 40 cycles of 95 °C/15 s and 60 °C/1 min. TaqMan primers and probes were designed to target mRNA sequences based on criteria set by Applied Biosystems. Probes were 5'-modified with 6-FAM and 3'-modified with TAMRA. Primer and probe sequences and target mRNA accession numbers are presented in Table 1. Intra- and inter-plate coefficients of variation (CV) for each Q-PCR assay (ActRI, ActRIIA, ActRIIB, LHR, FSHR, betaglycan and GAPDH) were between 0.9–1.5% and 7.4–10.2% respectively. Intra-assay CV values were based on independent ($n=30$) Ct sample values across a single Q-PCR plate, whereas inter-assay CVs were based on the calculated concentrations of pre-aliquoted quality control samples tested on independent Q-PCR plates ($n=4$).

Immunoassays

Cell-conditioned media (CM) were assayed for InhA using a specific two-site ELISA that uses monoclonal antibodies raised against synthetic peptide fragments of the human α - and β A-subunits (Muttukrishna *et al.* 1994). This assay has been validated for domestic fowl as described by Lovell *et al.* (1998). Recombinant human InhA was used as an assay standard (detection limit 2 pg/well). The ELISA showed minimal (<0.3%) cross-reaction with bovine inhibin α -subunit (200 ng/ml), rh inhibin-B (10 ng/ml), activin-A (10 ng/ml), follistatin (20 ng/ml). When tested at 1, 10 and 100 ng/ml, the ovine FSH and LH preparations used in the study showed no detectable cross-reaction (<0.002%) in the inhA ELISA.

Table 1 TaqMan primer and probe sequences used in the study.

Target	Forward primer 5' to 3'	Reverse primer 5' to 3'	TaqMan probe 5' (6-FAM) to 3' (TAMRA)	Accession number
Activin type I receptor	ccggaggcttgacgaaa	gcccagatcgaccctcttg	catccaggcagactgcttcgactcc	AJ318064
Activin type IIA receptor	tcacgaaaagggttcattaactga	ctgagcaatgtgacacagctcat	ttcctcaaggctaactggttccctgg	D31899
Activin type IIB receptor	tccctgcttaacatcctgtgta	ccaagaggatggccactga	tctctgctgcccacgctgtctct	U31223
Betaglycan	atgaagcttgacactctctgaat	gacaagaggcttggtgaagggtt	tggatgatcttgccatgatgcaca	L01121
LH receptor	ttcttcaacggcactggagtc	tccaggtctcaatgcaatg	ctgcctagctatggcctggaggcc	NM204936
FSH receptor	ccgggacattcccacca	gatgagagtcattccgaaggga	tgccacagaactgagattgtcctcacca	NM205079
GAPDH	ggagtcacacggatttgcc	tttgccagagaggacggc	tattggcgcctggtcaccagg	K01458

P4 and oestradiol (E2) concentrations were determined by direct RIA as described by Sauer *et al.* (1986) and Tannetta *et al.* (1998) respectively. The detection limits of the assays were 8 and 1.5 pg/ml respectively. Intra- and inter-assay coefficients of variation for each assay were <10%. Attempts were made to quantify secretion of InhB, ActA and FS in selected GC and TC culture experiments (Methods as described in Lovell *et al.* 1998, 2000, 2003). However, levels were below the detection limit of each assay (15 pg/well, 50 pg/well and 0.6 ng/well respectively).

Statistical analysis

One-way ANOVA was used in conjunction with *post hoc* Fisher's protected least significant difference (PLSD) test to evaluate treatment effects on levels of mRNA encoding receptors and on hormone release by each of the different follicle GC and TC populations (6–8 mm and F1 follicle). Two-way ANOVA was used to make comparisons between the effects of treatments on the different cell populations. Levels of mRNA for each receptor were normalised to GAPDH. A value of $P < 0.05$ was considered significant. There was no significant difference (ANOVA; $P > 0.05$) in GAPDH mRNA transcript levels between the GC and the TC cultures of different follicle classes or following treatment with LH or FSH. Hormone secretion results presented are for the final 24 h period of culture. In a given experiment, treatments were tested using triplicate wells which were pooled for RNA isolation within a given culture and each experiment was repeated using at least three independent cultures (utilising independently isolated cell preparations). All values presented are means $\pm$ S.E.M. (with n indicating the number of independent cultures).

Results

The expression of mRNAs for the three ActRs (ActR-I, IIA, -IIB), β gly, FSH-R and LH-R was readily detectable in the GC and TC from both follicle stages under investigation (6–8 mm and F1). In contrast, oestradiol was only detectable in TC-CM, whereas P4 was predominantly produced by the F1 GC.

Effect of LH and FSH on mRNA for ActRI, ActRIIA, ActRIIB, β gly, LH-R and FSH-R in GCs from 6 to 8 mm follicles (6-8GC)

Changes in the 6-8GC expression of mRNAs encoding ActRs (ActR-I, -IIA, -IIB) and β gly during culture with LH or FSH are shown in Fig. 1. LH treatment (100 ng/ml) caused a significant ($P < 0.05$; 83%) increase in ActRI mRNA transcript level, whereas FSH (100 ng/ml) promoted a decrease ($P < 0.05$; 65%). FSH (10–100 ng/ml;

Figure 1 Effects of LH and FSH on mRNA levels for ActRI, ActRIIA, ActRIIB, β gly, FSH-R and LH-R in granulosa cells isolated from 6–8 mm ovarian follicles. Values are means $\pm$ S.E.M. ($n = 3$ –5 independent cultures) and means without a common letter (case-specific) are significantly ($P < 0.05$) different (by ANOVA and Fisher's PLSD test). Upper and lower case letters show a significant difference with LH treatment (black columns) and FSH treatment (white columns) respectively.

3.7–8.5-fold) and LH (100 ng/ml; 8.5-fold) significantly increased the ActRIIA mRNA level. Changes in ActRIIB mRNA with LH and FSH treatment paralleled ActRI transcript; 100 ng/ml LH increased significantly ($P < 0.01$; 3.7-fold), while 100 ng/ml FSH decreased significantly ($P < 0.05$; 57%) ActRIIB transcript. β gly mRNA level was significantly ($P < 0.01$) increased by LH (10–100 ng/ml; by up to twofold) and FSH (1–100 ng/ml; by up to 3.5-fold). FSH (10–100 ng/ml) significantly increased (4.5-fold; $P < 0.0001$) the LH-R mRNA expression in 6-8GC. All doses of FSH increased FSH-R transcript (eight- to tenfold) when compared with controls. LH also caused a dose-dependent increase in FSH-R mRNA expression (up to eightfold).

Effect of LH and FSH on mRNA for ActRI, ActRIIA, ActRIIB, β gly, LH-R and FSH-R in TCs from 6 to 8 mm follicles (6-8TC)

Changes in the 6-8TC expression of mRNAs encoding ActRs (ActR-I, -IIA, -IIB) and β gly during culture with LH or FSH are shown in Fig. 2. Neither LH nor FSH significantly altered the mRNA expression of ActRI, ActRIIA or ActRIIB ($P > 0.05$). However, both LH and FSH significantly ($P < 0.05$)

Figure 2 Effects of LH and FSH on mRNA levels for ActRI, ActRIIA, ActRIIB, β gly, FSH-R and LH-R in theca cells isolated from 6–8 mm ovarian follicles. Other details are the same as given in the legend to Fig. 1.

increased the β gly transcript level (7- and 3.5-fold respectively). FSH-R mRNA expression in 6-8TC was stimulated by both LH (threefold) and FSH (fourfold) at 100 ng/ml. LH and FSH also stimulated LH-R transcript levels in 6-8TC (5.6- and 4.7-fold respectively).

Effect of LH and FSH on mRNA for ActRI, ActRIIA, ActRIIB, β gly, LH-R and FSH-R in GCs from F1 follicles (F1 GC)

Changes in the levels of mRNAs encoding ActRs and β gly in the F1 GC are shown in Fig. 3. ActRI mRNA expression was increased by both LH (10–100 ng/ml; twofold) and FSH (100 ng/ml; 2.4-fold). ActRIIA transcript level was not significantly altered by LH or FSH treatment. ActRIIB mRNA expression was increased dose-dependently by FSH (up to 2.7-fold; $P < 0.01$), whereas only 100 ng/ml LH significantly increased expression (3.2-fold). β gly mRNA level was also significantly ($P < 0.001$) increased by LH (100 ng/ml; sevenfold) and FSH (100 ng/ml; fourfold). The expression of LH-R mRNA in F1 GC was increased approximately twofold by LH (10–100 ng/ml) while FSH had no significant effect. In contrast, FSH-R mRNA expression was slightly increased by FSH (40%; 100 ng/ml; $P < 0.05$) while LH had no effect.

Figure 3 Effects of LH and FSH on mRNA levels for ActRI, ActRIIA, ActRIIB, β gly, FSH-R and LH-R in granulosa cells isolated from the largest (F1) preovulatory follicle. Other details are the same as given in the legend to Fig. 1.

Effect of LH and FSH on mRNA for ActRI, ActRIIA, ActRIIB, β gly, LH-R and FSH-R in TCs from F1 follicles (F1 TC)

Changes in the F1 TC expression of mRNAs encoding ActRs (ActR-I, -IIA, -IIB) and betaglycan are shown in Fig. 4. Neither LH nor FSH affected the expression of ActRI and ActRIIB mRNA ($P > 0.05$) although they slightly reduced the ActRIIA mRNA expression by 35–50% ($P < 0.01$). LH and FSH dose-dependently increased β gly transcript level by 4.5-fold ($P < 0.05$) and 7.6-fold ($P < 0.0001$) respectively. In F1 TC, unlike in F1 GC, expression of FSH-R transcript was increased twofold by LH (at 100 ng/ml) but FSH had no effect. In contrast, LH-R mRNA expression in F1 TC was increased by both LH (up to fivefold) and FSH (up to 2.5-fold).

Comparison between prehierarchal and preovulatory follicles: basal and gonadotrophin-induced expression of mRNA for ActR and β gly

Two-way ANOVA of ActRI and ActRIIB transcript data revealed that GC from the two follicle types responded very differently to FSH treatment ($P < 0.0001$); whereas FSH promoted a significant reduction in mRNA

Figure 4 Effects of LH and FSH on mRNA levels for ActRI, ActRIIA, ActRIIB, β gly, FSH-R and LH-R in theca cells isolated from the largest (F1) preovulatory follicle. Other details are the same as given in the legend to Fig. 1.

expression in the 6-8GC, transcript levels were increased in the F1 GC. No corresponding difference was found in the GC response to LH for these two receptors ($P > 0.05$, two-way ANOVA), although ActRI mRNA expression was significantly higher in the F1 GC than in 6-8GC ($P < 0.05$). LH and FSH had no effect on thecal ActRI and ActRIIB mRNA expression; however, levels of ActRIIB transcript was significantly higher in the F1 TC than in 6-8TC (two-way ANOVA).

ActRIIA mRNA expression in both GC and TC was significantly higher in the F1 than in 6-8 mm follicles. However, only 6-8GC ActRIIA mRNA expression was significantly increased by LH and FSH ($P < 0.0001$). In contrast, ActRIIA mRNA expression in the F1 TC, but not 6-8 mmTC, was significantly ($P < 0.01$) reduced by LH and FSH treatment.

β gly mRNA expression in 6-8GC + TC and F1 GC + TC was positively regulated by LH and FSH. The levels of β gly transcript were maximal with LH in F1 GC and with FSH in 6-8GC. The β gly mRNA expression in F1 TC was stimulated by LH to a lesser extent than FSH (4.5-fold vs 7-fold), while in 6-8TC LH was more stimulatory than FSH (7.2-fold vs 3.5-fold; $P < 0.01$).

Analysis using multifactorial ANOVA demonstrated that TC β gly mRNA expression within 6-8 mm follicles was significantly higher ($P < 0.05$) than in the

corresponding GC following LH and FSH stimulation. F1 TC β gly mRNA expression was also significantly higher than in the corresponding GC following FSH stimulation, although there was no significant difference following LH stimulation.

Effect of LH and FSH on the release of InhA, P4 and E2 by 6-8 mm and F1 GC and TC

The release of InhA, P4 and E2 in response to LH and FSH treatment in 6-8 mm and F1 GC and TC is shown in Fig. 5. Basal InhA levels in 6-8GC media were below the assay detection limit (2 pg/ml); however, following stimulation by LH (all doses) and FSH (10–100 ng/ml), InhA levels increased to

Figure 5 Effects of LH and FSH on the secretion of inhibin A, progesterone and oestradiol by cultured cells derived from (A) theca and (B) granulosa cells from 6-8 mm follicles and (C) theca and (D) granulosa cells from the largest (F1) follicle. Thecal inhibin A and granulosa oestradiol secretion (basal and stimulated) are not plotted as values were below the assay detection limit in both the 6-8 mm and F1 follicle classes. The dashed line represents the detection limit of the assay. Other details are the same as given in the legend to Fig. 1.

detectable levels (Fig. 5B). LH and FSH both elicited marked dose-dependent increases in P4 release by 6-8GC (5.6-fold and 17.3-fold respectively; $P < 0.001$). Conversely, LH promoted a greater P4 increase than FSH in F1 GC (5-fold vs 1.5-fold; $P < 0.05$ by two-way ANOVA). It should be noted that basal P4 release was some 1000 times greater for F1 GC when compared with 6-8GC. LH and FSH treatment of F1 GC also significantly increased InhA release (up to fourfold; $P < 0.05$). E2 was undetectable in GC-CM under all treatment conditions.

Treatment of 6-8TC with LH or FSH dose-dependently increased ($P < 0.001$) E2 release up to 46-fold and 73-fold respectively (Fig. 5A). E2 release by F1 TC was much lower than in 6-8TC and only a slight response to FSH was observed (Fig. 5C). Basal P4 levels in both 6-8TC and F1 TC CM were at or below the assay detection limit (8 pg/ml) but rose to detectable levels following LH or FSH treatment. As in F1 GC, FSH had no effect on P4 release in F1 TC. It should be noted that basal- and LH-stimulated P4 release from F1 GC were ~6500-fold and 15 000-fold respectively, higher than from F1 TC.

Discussion

Consistent with immunocytochemical evidence that ActRI, ActRIIA, ActRIIB and β gly proteins are expressed in GC from hen ovarian follicles (Lovell *et al.* 2002a, Sweeney & Johnson 2005), the present study shows that mRNA transcripts for ActRI, ActRIIA, ActRIIB and β gly are detectable within cultured GC and TC derived from both prehierarchical (6–8 mm) and preovulatory F1 follicles. Moreover, we demonstrate that pituitary gonadotrophins (LH and FSH) modulate these receptor transcripts within GC and TC at both follicle stages.

A quantitative assessment of receptor protein expression under the different experimental conditions was beyond the scope of this study but previous *in vitro* studies on chicken GCs clearly support the presence of functional type-I and -II ActRs that are capable of forming active signalling complexes. Lovell *et al.* (2002a) shown that ActA (β A- β A dimer, which is mainly confined to TC in chickens; Lovell *et al.* 1998, 2003) enhanced gonadotrophin-induced secretion of InhA (α - β A dimer) and P4 by GC from hen preovulatory follicles. ActA also enhanced FSH-R and LH-R mRNA expression in GC from preovulatory follicles (Johnson *et al.* 2006). An ActA-induced increase in FSH-R mRNA expression has also been observed in GC from prehierarchal hen follicles (Johnson *et al.* 2004, 2006, Woods & Johnson 2005).

Within the hen GC layer, there is a developmental shift from an FSH- to an LH-dependent mechanism of regulating follicular development as follicles are selected (Tilly *et al.* 1991, Johnson *et al.* 1996, You *et al.* 1996), similar to that occurring in mammals

(Richards 1994). Of key interest in this regard are the findings that FSH reduced and LH increased both ActRIIB and ActRI mRNA transcript levels within GC of 'unselected' prehierarchical (6–8 mm) follicles, whereas both LH and FSH stimulated mRNA expression of both receptors in 'selected' F1 GC. We hypothesise that it is this shift in the response to FSH and LH of ActR mRNA expression that modulates this responsiveness of cells to intrafollicular activin.

As it has been shown that ActA increased LH-R mRNA expression in 6–8 mmGC (Johnson *et al.* 2004), the present and previous data lead us to propose that GC of prehierarchical follicles approaching selection, which are primarily FSH-driven, have reduced expression of key ActRs (ActRI and ActRIIB) and therefore a diminished responsiveness to Act. In turn, as follicles are selected and become more responsive to LH, ActRI and ActRIIB mRNA expression is upregulated leading to greater co-stimulation by Act. Indeed, ActA can further stimulate LH-R mRNA expression in 6–8 mm GC (Johnson *et al.* 2004), which could lead to a further increase in LH-responsiveness aiding 'selection'.

Once selected, GC from preovulatory follicles primarily secrete InhA and P4. This secretion in response to ActA is greatly enhanced during co-treatment with LH or FSH (greater than the sum of the responses to ActA and LH/FSH alone; Lovell *et al.* 2002a); the present data suggest that this increased responsiveness to Act may in part be due to a LH/FSH stimulated increase in ActR. Consistent with this and with the above-mentioned functional evidence of synergism between ActA and gonadotrophins, ActA was recently shown to upregulate the expression of LH-R and FSH-R mRNA in preovulatory follicles (Johnson *et al.* 2006).

Although LH and FSH differentially regulate ActR and β gly transcript levels (this study) and, reciprocally, ActA can increase LH-R and FSH-R mRNA expression in prehierarchical follicle GC (Johnson *et al.* 2004, 2006), further work is required to understand how the balance of these, and most likely other, endocrine and paracrine signals allow the promotion of one 'selected' follicle into the preovulatory hierarchy.

GC inhibin B (InhB; α - β B dimer) protein levels (Lovell *et al.* 2003) peak in 6–8 mm prehierarchical follicles before falling steadily from the stage of follicle selection. Although InhB can associate with the ActRIIs, association with co-receptor β gly, which is also expressed (Sweeney & Johnson 2005, Lovell *et al.* 2006) can greatly enhance their affinity for ActRIIs (Lewis *et al.* 2000). However, as granulosa β gly mRNA expression (in this study) was an order of magnitude lower in prehierarchical follicles when compared with preovulatory follicles, InhB/ β gly may not be as an effective Act antagonist on prehierarchical GCs as InhA/ β gly in preovulatory follicles. Unfortunately, InhB protein levels in GC-CM from 6–8 mm follicles were below the current assay detection limits. In a previous study, however, we were

able to detect InhB in CM from 6–8 mm follicle wall explant cultures (Lovell *et al.* 2003). This suggests that a theca-derived factor present in follicle wall explant cultures (and absent in the GC mono-cultures) may play a role in upregulating InhB production. ActA is a prime candidate here as ActA treatment increased inhibin/activin β B-subunit mRNA level in GC from 6–12 mm follicles (Johnson *et al.* 2006). Further work is required to establish whether this increase in β B-subunit mRNA leads to increased InhB secretion although, as mentioned above, this is technically challenging due to the limited sensitivity of the current inhibin B assay.

InhA protein levels were below the assay detection limits in 'basal' CM from 6–8 mm GC (this study) and

6–8 mm follicle wall explants (Lovell *et al.* 2003) and in GC extracts from follicles <9mm (Lovell *et al.* 2003). Following gonadotrophin stimulation of 6–8 mm GC (this study) InhA secretion increased to detectable levels (albeit very much lower than from F1 GCs). The functional significance of the dramatic shift from InhB to InhA production around the point of follicle selection *in vivo* (Lovell *et al.* 2003) remains obscure and further work is warranted to investigate this striking phenomenon. Previous data in mammals indicate that β gly potentiates the binding of both InhA and InhB to ActRIIA, with the complex being resistant to disruption by ActA. In contrast, only InhA binding to ActRIIB was significantly enhanced by β gly (Chapman *et al.* 2002).

Figure 6 Proposed model showing events related to the regulation of Act signalling within the laying hen ovary. (A) In primarily FSH-responsive pre-hierarchical (6–8 mm) follicles FSH reduces ActRIIB (IIB) and ActRI (I) whilst stimulating ActRIIA (IIA) and β Gly mRNA expression. High levels of granulosa-derived InhB can complex with IIA/ β Gly, blocking thecal-derived ActA signalling. ActA action is further blocked by binding to FS (Schneyer *et al.* 2002). Upon acquiring LH-responsiveness, LH increases IIB and I mRNA expression allowing ActA to stimulate transcription through IIB and I via Smad2 (Johnson *et al.* 2004) as, unlike with IIA, ActA can compete with InhB/ β Gly for binding with IIB (Chapman *et al.* 2002). (B) Following transition to preovulatory follicle status, InhA rather than InhB is produced by GC and there is an increase in ActA secretion without a rise in FS (Lovell *et al.* 2003). LH stimulation also increases I, IIB and β Gly mRNA expression. ActA signalling is therefore regulated by complexing with FS and availability of I, IIA and IIB receptors, where IIA can be sequestered by binding with Inh/ β Gly.

Therefore, it is tempting to speculate that, at the stage of follicle selection (~6–8 mm) when granulosa InhB levels peak and InhA levels have yet to rise (Lovell *et al.* 2003), access of thecal-derived ActA to granulosa ActRIIB receptors would not be antagonised, allowing recruitment of ActRI to generate an active signalling complex (see Fig. 6).

In F1 GC, the expression of β gly and ActRIIB mRNA was significantly increased by LH and FSH treatment. InhA release was also significantly higher in F1 GC than in 6-8GC, reflecting *ex vivo* data (Lovell *et al.* 2003). Unlike ActA, InhA treatment alone has no effect on FSH-R or LH-R mRNA expression in F1 GC (Johnson *et al.* 2006) supporting the notion that GC-derived InhA acts through antagonism of Act signalling by association with β gly/ActRII.

Although only one ActRI subtype to date has been cloned in birds and investigated in this study (Accession number AJ318064), it does not exclude the possibility that multiple ActRI subtypes exist, as in mammals (Attisano *et al.* 1993, Tsuchida *et al.* 1995) or that ActRIIs may also form signalling complexes with related TGF β -superfamily type-I receptors.

As with all studies investigating mRNA expression, the possibility cannot be excluded that the observed changes in steady-state mRNA transcript levels reflect changes in mRNA stability, rather than effects on gene transcription *per se*. Further, studies to verify that the changes in ActR and β gly mRNA transcript levels reported here are accompanied by alterations in receptor protein expression would also be useful. However, as discussed earlier, this seems highly likely considering previous functional studies (Lovell *et al.* 2002a, Johnson *et al.* 2004, 2006, Woods & Johnson 2005) which demonstrated interactions between the effects of ActA and gonadotrophins on hen GCs.

In conclusion, this study shows, for the first time in an avian species, that mRNA transcript levels for ActR and β gly in TC and GC are modulated by gonadotrophins according to the cell-type and stage of follicle development. These findings, together with emerging evidence that follicles express a myriad of ligands, receptors and binding proteins, underscore the complexity of potential interactions between systemic and locally produced factors required to coordinate follicle progression into and through the preovulatory hierarchy.

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