Differential expression of mRNAs encoding the putative inhibin co-receptor (betaglycan) and activin type-I and type-II receptors in preovulatory and prehierarchical follicles of the laying hen ovary

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Abstract

Ovarian follicle development is primarily regulated by an interplay between the pituitary gonadotrophins, LH and FSH, and ovary-derived steroids. Increasing evidence implicates regulatory roles of transforming growth factor-β (TGFβ) superfamily members, including inhibins and activins. The aim of this study was to identify the expression of mRNAs encoding key receptors of the inhibin/activin system in ovarian follicles ranging from 4 mm in diameter to the dominant F1 follicle (~40 mm).

Ovaries were collected (n=16) from mid-sequence hens maintained on a long-day photoschedule (16 h of light:8 h of darkness). All follicles removed were dissected into individual granulosa and thecal layers. RNA was extracted and cDNA synthesized. Real-time quantitative PCR was used to quantify the expression of mRNA encoding betaglycan, activin receptor (ActR) subtypes (type-I, -IIA and -IIB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH); receptor expression data were normalized to GAPDH expression. Detectable levels of ActRI (granulosa: r=0·49, P<0·001, n=144/group; theca: r=0·49, P<0·001, n=144/group) was well correlated. No significant correlations were identified between betaglycan and ActRIIA or -IIB. Considering all follicles analysed, granulosa mRNA expression of betaglycan, ActRI, ActRIIA and ActRIIB were all significantly lower than in corresponding thecal tissue (betaglycan, 11·4-fold; ActRIIB, 5·1-fold; ActRI, 3·8-fold; ActRIIA, 2·8-fold).

In all follicles studied expression of betaglycan and ActRI (granulosa: r=0·65, P<0·001, n=144/group; theca: r=0·49, P<0·001, n=144/group) was well correlated. No significant correlations were identified between betaglycan and ActRIIA or -IIB. Considering all follicles analysed, granulosa mRNA expression of betaglycan, ActRI, ActRIIA and ActRIIB were all significantly lower than in corresponding thecal tissue (betaglycan, 11·4-fold; ActRIIB, 5·1-fold; ActRI, 3·8-fold; ActRIIA, 2·8-fold). The co-localization of type-I and -II activin receptors and betaglycan on granulosa and thecal cells are consistent with a local auto/paracrine role of inhibins and activins in modulating ovarian follicle development, selection and progression in the domestic fowl.


Introduction

The mechanisms controlling ovarian follicular dynamics in the hen are complex and involve the interplay between neuroendocrine, endocrine, paracrine and autocrine signals. The pituitary gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are key regulators which have been shown to stimulate production of ovarian steroids (Tilly et al. 1991a,b) and peptides including inhibin and activin (Lovell et al. 2002a) in a development-related manner. As in mammals, inhibins and activins of gonadal origin have been implicated as regulators of follicle development and progression in the hen (for review see Knight et al. 2005).

Inhibins and activins are members of the structurally conserved but functionally diverse transforming growth factor-β (TGFβ) superfamily of extracellular signalling molecules (Knight & Glister 2003). Inhibins are dimeric glycoproteins consisting of an α-subunit disulphide linked to one of two distinct β-subunits (βA or βB), to generate inhibin A and inhibin B respectively. Activins are disulphide-linked homo- or hetero-dimers of βA and/or...
**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 under a standard long-day photo-schedule of 16 h of light and 8 h of darkness, at an ambient temperature of 21–23 °C. Food and water were freely available. Ovipositions were monitored using time-lapse recording technology and used to predict the time of ovulation.

**Recovery and preparation of follicle extracts**

Hens (n=16) were killed by cervical dislocation 4 h, 12 h, 18 h and 22 h after the predicted ovulation of a mid-sequence egg. Ovarian follicles of 4 mm in diameter and above were dissected from the ovary and their diameters recorded. All follicles were subsequently dissected into separate granulosa and thecal layers (Gilbert et al. 1977) which were washed in saline (0.75% (w/v)), snap frozen in solid CO₂ and stored at −70 °C.

Granulosa and thecal tissues from ≥10 mm follicles and six separate granulosa and thecal follicle pools from 4–5.9 mm, 6–7.9 mm and 8–9.9 mm follicles were weighed and homogenized in an ~15 times volume of Tri-reagent (Sigma) using an Ultra-Turrax T8 homogenizer (IKA, Staufen, Germany). RNA was subsequently purified as described in the standard Tri-reagent protocol. The final RNA pellet was resuspended in 100µl nuclease-free water (containing RNA Secure (Ambion, Huntington, Cambridgeshire, UK)), then treated with RNase-free DNase (15 min at 37 °C; RQ1; Promega). The purified RNA was re-purified using a 15 times volume of Tri-reagent. The resultant purified RNA was resuspended in 50µl nuclease-free water (containing RNA Secure).

RNA was quantified on a spectrophotometer (GeneQuant; GE Healthcare, Amersham) and cDNA was synthesized using ImProm-II reverse transcriptase (Promega; used according to the supplier’s 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 quantitative PCR reactions were carried out using 1 µl diluted RT reaction product or 1 µl standard (from 200 to 1:56 amol/µl), in a volume of 25 µl containing 12.5µl master mix with 1 µl ROX dye (Abgene, Epsom, Surrey, UK), 2 µl forward and reverse primers...
(see Table 1) were each added (final concentration: 50–900 nM), 1 µl probe (final concentration: 100–200 nM; see Table 1) and 5·5 µl nuclease-free water. 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/15s and 60°C/1 min. TaqMan primers and probes were designed to target mRNA sequence based on criteria set by Applied Biosystems.

Statistical analysis

One-way ANOVA was used in conjunction with post hoc Fisher’s protected least significant difference (PLSD) test to determine whether expression of mRNAs encoding receptors varied between different follicular size categories. Levels of expression of each mRNA detected were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. $P<0.05$ was considered to be significant. The means are ± S.E.M. Preliminary assessment of the follicular gene expression results obtained from hens killed at the four different time-points during the ovulatory cycle ($n=4$ hens per time-point) revealed a high degree of individual variation; results for all 16 hens were therefore combined for further analysis and presentation.

Results

Granulosa cell expression of mRNA for ActRI, ActRIIA and ActRIIB

Changes in granulosa expression of mRNAs encoding ActRI, ActRIIA and ActRIIB during follicle development are shown in Fig. 1. Expression of the three activin receptor subtypes was measurable in all follicle size classes studied (4 mm–F1). ActRI mRNA expression remained at a similar level from 4–7.9 mm (43.2 ± 4.5 amol/fmol GAPDH); levels increased significantly ($P<0.05$) to a peak at 8–9.9 mm (101.7 ± 27.7 amol/fmol GAPDH) before falling in the F6 follicles (31.0 ± 6.2 amol/fmol GAPDH). Expression levels in F6–F1 follicles were not significantly different from those in 4–7.9 mm follicles. Considering all follicle size classes, granulosa ActRI mRNA expression was 5–to 20-fold higher than ActRIIB expression. However, there was no significant difference in granulosa ActRIIB mRNA expression throughout follicular development (4.4 ± 0.59 amol/fmol GAPDH, $n=144$ group). ActRIIA expression level was similar to ActRI expression...
in 4–7·9 mm follicles (51·5 ± 9·6 amol/fmol GAPDH); ActRIIA levels rose significantly in the 8–9·9 mm class (121·6 ± 26·8 amol/fmol GAPDH) before falling progressively up to F2 (8·99 mm vs F2, \(P<0·05\)). Expression then rose sharply from F2 to F1 (3-fold; 53·7 ± 15 vs 161·4 ± 51 amol/fmol GAPDH, \(P<0·05\)). Overall, ActRIIA mRNA expression was 2·5- to 6-fold higher than ActRI expression in preovulatory granulosa cells. ActRIIA expression was well correlated with ActRIIB expression in the granulosa of the F1 follicles (\(r=0·664, P<0·01, n=16/\text{group}\)) and weakly correlated throughout follicle development (\(r=0·24, P<0·05, n=144/\text{group}\)).

**Thecal cell expression of mRNA for ActRI, ActRIIA and ActRIIB**

Changes in thecal expression of mRNAs encoding ActRI, ActRIIA and ActRIIB during follicle development are shown in Fig. 2. All activin receptor subtypes were measurable in all follicle size classes studied (4 mm–F1).

ActRI mRNA expression was similar from 4–7·9 mm (160·5 ± 17·9 amol/fmol GAPDH); levels decreased significantly from 6–7·9 mm to a nadir at the F4 position (97·9 ± 12·0 amol/fmol GAPDH, \(P<0·05\)). Expression then rose significantly (\(P<0·05\)) to the F1 (189 ± 36·8 amol/fmol GAPDH). Thecal ActRIIA mRNA expression was not significantly different from 4 mm to F2 (180·6 ± 44·4 to 262·5 ± 73·9 amol/fmol GAPDH); however, expression increased 2-fold from F2 to F1 (521·9 ± 173·4 amol/fmol GAPDH, \(P<0·05\)). Thecal ActRIIB expression was not significantly different from 4 mm to F3 (16·6 ± 4·6 to 22·3 ± 5·3 amol/fmol GAPDH) although expression significantly increased 2-fold from F3 to F1 (46·5 ± 11·2 amol/fmol GAPDH, \(P<0·05\)). ActRIIA mRNA expression was correlated with ActRIIB expression in F1 (\(r=0·783, P<0·01, n=16/\text{group}\)) and with ActRIIB (\(r=0·521, P<0·01, n=144/\text{group}\)) and ActRI (\(r=0·229, P<0·05, n=144/\text{group}\)) expression throughout follicle development. Relative to ActRI expression, the relative expression of ActRIIA increased ~3-fold from F6 to F1. Thecal ActRIIB expression was significantly lower than both ActRIIA (7- to 20-fold) and ActRI (4- to 14-fold) at all follicle stages analysed.

**Granulosa and thecal cell expression of mRNA for betaglycan**

Changes in granulosa and thecal expression of mRNA encoding betaglycan during follicle development are shown in Fig. 3. Betaglycan was measurable in the
granulosa and thecal layers of all follicle size classes studied (4 mm–F1). Granulosa betaglycan mRNA expression increased 3-fold from 4–5·9 mm (0·5 ± 0·1 amol/fmol GAPDH) to a peak at 8–9·9 mm (1·6 ± 0·4 amol/fmol GAPDH). Levels then fell as follicles progressed through the preovulatory hierarchy, being ~4-fold lower at the F3 and F2 positions (F2; 0·43 ± 0·18 amol/fmol GAPDH). Granulosa expression then peaked abruptly (P<0·05) in F1 (4-fold increase; 1·4 ± 0·41 amol/fmol GAPDH). In contrast, thecal betaglycan expression remained relatively high in the prehierarchical follicles (4–8·9 mm; 12·1 ± 1·4 amol/fmol GAPDH, n=48) before falling ~2-fold in the F6 position (6–7·9 mm vs F6, P<0·05). Betaglycan mRNA expression increased through the preovulatory hierarchy to the F2 position (F6 vs F2; P<0·05), before falling significantly from F2 to F1 (14·5 ± 3·4 vs 8·1 ± 2·4 amol/fmol GAPDH). Betaglycan mRNA expression in the theca was between 4·5-fold (F6) and 34-fold (F2) higher than in the corresponding granulosa layer.

**Discussion**

The present study used quantitative PCR (TaqMan) to examine for the first time the spatio-temporal pattern of expression of mRNAs encoding activin receptors (ActRI, ActRIIA and ActRIIB) and betaglycan throughout folliculogenesis in an avian species, although recent studies have confirmed that betaglycan is expressed in ovarian follicles (Sweeney & Johnson 2005) and that ActRIIA is expressed in the broiler hen ovary (Slappey & Davis 2003).

Our results have shown detectable expression of mRNA for betaglycan, ActRI, ActRIIA and ActRIIB within the granulosa and thecal layers of all follicle stages analysed, with mRNA expression for all receptors much higher in the thecal than in the granulosa layer. These differences may reflect differential steroidogenic potential and/or inhibin-related protein production (Etches & Duke 1984, Lovell et al. 1998, 2003). Thecal cells produce steroids at all stages of follicle development but granulosa cells from prehierarchical (3–8 mm) hen follicles are steroidogenically incompetent (Tilly et al. 1991b). Inhibin A is also undetectable in granulosa cells of follicles <9 mm although they produce inhibin B, activin A and follistatin (Lovell et al. 2003). The action of activin would be greatest when follistatin levels are low. Similarly, since inhibins antagonize the actions of activin, low levels of inhibin should also favour activin action. As shown previously (Lovell et al. 2003) follicular activin A content was relatively uniform in follicles ranging from 1 to 9 mm while follistatin content increased progressively from 1 to 7 mm, suggesting a gradual attenuation of ‘activin tone’. The surge in inhibin B production in follicles between 5 mm and 8 mm would further diminish ‘activin tone’ relative to ‘inhibin tone’ at this stage.

The primary source of oestrogen in the hen ovary is the thecal layer of small prehierarchical follicles (Senior & Furr 1975, Robinson & Etches 1986) which, as reported here, have detectable mRNA expression of all activin receptor subtypes. Furthermore, addition of oestradiol to cultured laying hen granulosa cells from small yellow follicles and large white follicles increased follistatin and significant correlations were identified between betaglycan and ActRIIA or ActRIIB when analysing the granulosa or thecal layers independently. Considering all follicle stages, there was a positive correlation between granulosa and thecal ActRIIB mRNA expression (r=0·45, P<0·001, n=144/group) with particularly high correlations between the granulosa and thecal layer in F1 (r=0·69, n=16/group), 8–9·9 mm (r=0·74, n=16/group), 6–7·9 (r=0·86, n=16/group) and 4–5·9 mm (r=0·65, n=16/group) follicles. Betaglycan mRNA expression in granulosa and theca was highly correlated in the 6–7·9 mm follicle category (r=0·88, P<0·001, n=16/group) but not at other follicle stages.

**Relationship among ActRI, ActRIIA and ActRIIB and betaglycan in ovarian follicles**

Considering the combined data for all classes of follicle stages analysed (4 mm–F1; see Fig. 4), granulosa expression of betaglycan, ActRI, ActRIIA and ActRIIB mRNAs were all significantly lower (as expressed relative to GAPDH) than in corresponding thecal tissue (betaglycan, 11·4-fold; ActRIIB, 5·1-fold; ActRI, 3·8-fold; ActRIIA, 2·8-fold). In all follicles studied there was a good correlation between mRNA expression of betaglycan and ActRI (granulosa: r=0·65, P<0·001, n=144/group; theca: r=0·50, P<0·001, n=144/group). No
activin βB-subunit mRNA expression, with no increase in immunoreactive α-subunit protein (Davis et al. 2000), suggesting that theca-derived oestrogen may regulate granulosa inhibin B, or possibly activin B production in prehierarchical follicles.

In the avian ovary, one prehierarchical 6–8 mm follicle is ‘selected’ daily to enter the preovulatory follicle hierarchy from approximately six to ten visually similar follicles. Selection is thought to be governed by sensitivity to FSH, with the highest FSH receptor (FSH-R) mRNA content detected in this class (You et al. 1996). In contrast to observations in mammals (Nakamura et al. 1993), activin A was unable to induce FSH-R mRNA expression in cultured hen granulosa cells from 6–8 mm follicles (Woods & Johnson 2005). This may be due to endogenous follistatin production neutralizing the activin A treatment and immunoneutralization of endogenous follistatin or inhibin together, regulate FSH-R expression. Incubation of 6–8 mm follicle granulosa cells with FSH renders these follicle population. It is suggested that exogenous activin A treatment and immunoneutralization of endogenous follistatin or inhibin α-subunit peptide (amino acids 1–26). At 26 weeks, IMM hens showed a 2-fold increase in the number of 8–9.9 mm follicles. This size class normally corresponds to follicles around the point of entry to the preovulatory follicle hierarchy; they also show a peak in inhibin B production (Lovell et al. 2003). Whether IMM altered expression of ActRs and/or betaglycan, thus changing follicular responsiveness, needs further evaluation. However, the absence of significant changes in plasma LH and FSH after inhibin immunization (Lovell et al. 2001) and the absence of changes in plasma FSH during the laying hen ovulatory cycle (Lovell et al. 2000) suggest that inhibin may reduce the FSH responsiveness of this follicle population. It is suggested that exogenous activin A treatment and immunoneutralization of endogenous follistatin or inhibin α-subunit peptide raises ‘activin tone’ relative to ‘inhibin tone’, allowing normally unselected follicles with a higher inhibin:activin ratio and/or betaglycan expression to be selected into the hierarchy. This concept is reinforced by the present finding of a peak in mRNA expression for ActRIIA, ActRI and betaglycan in the granulosa layer of 8–9.9 mm follicles.

This leads us to hypothesize that one ‘privileged’ follicle of the growing small yellow follicle (SYF) cohort is promoted to the preovulatory hierarchy each cycle by virtue of its having an intrinsically high ‘activin tone’ and significant expression of ActRI and ActRII, with a low ‘inhibin tone’ and/or low level of betaglycan. In this study there was always a strong positive correlation between ActRI and betaglycan, suggesting that the balance between activin and inhibin signalling components is tightly regulated. In vivo it is likely that a cocktail of factors, including FSH, activin, follistatin and inhibin together, regulate FSH-R expression. Incubation of 6–8 mm follicle granulosa cells with FSH renders these follicle responsive to LH stimulation (Tilly et al. 1991b) with increased progesterone production (Li & Johnson 1993a, b). Preliminary attempts to identify such ‘privileged’ 6–9 mm follicles by examining basal gonadotrophin-induced hormone secretion by individual follicle wall explants in vivo have yielded inconclusive results (authors’ unpublished observations). However, Woods & Johnson (2005) recently detected individual follicles in the 6–9 mm cohort expressing elevated FSH-R mRNA expression. Whether these individual follicles have a high ‘activin tone’ and/or elevated ActRIs with low inhibin and/or betaglycan expression remains to be determined.

Expression of activin receptors has also been identified in mammalian ovaries with ActRIIA and ActRIIB protein in early follicles from the cow (Hulshof et al. 1997) and ActRIIA, ActRIIB, ActRIA and ActRIB in the rat (Drummond et al. 2002). The present study identified ActRIIB mRNA as being the least abundant of the activin subtypes; this was also demonstrated in the postnatal rat ovary (Drummond et al. 2002). However, although ActRIIB mRNA expression is lower than ActRIIA during follicle development it may still contribute a higher proportion of activin receptor protein than expression suggests. In vitro studies have shown that activin A can stimulate early follicle development in cows (Hulshof et al. 1997), sheep (Thomas et al. 2003) and rodents (Liu et al. 1998, Smitz et al. 1998, Zhao et al. 2001), implicating it as a modulator of early follicular development in mammals. Factors regulating expression of ActRI, ActRIIA, ActRIIB and betaglycan in the hen ovary have yet to be explored; however, expression of ActRIA and ActRIIA is regulated by gonadotrophins and oestradiol in adult rats (Aloi et al. 1997).

Lovell et al. (2003) showed that transition into the preovulatory hierarchy was associated with a marked rise in thecal activin A (~8-fold rise; 8 mm–F4) and a decline in the follistatin (~5-fold) and inhibin B (~27-fold) content of the granulosa layer from 8 mm to F1 (Lovell et al. 2003). This increase in activin A, decrease in inhibin A or decrease in follistatin, or any combination would enhance the paracrine signalling of theca-derived activin A through type-I and -II activin receptors expressed on granulosa tissue. As shown here, ActRIIA mRNA levels fell progressively from follicle selection to F2 with no significant change in ActRI expression from F6 to F1. Whether the reduction in ActRIIA expression reduces activin-stimulated events is unknown; however, the reduction in granulosa betaglycan expression (between 8 mm and F2) and the increase in ‘activin tone’ would be expected to facilitate activin function.

In hens, LH receptor (LH-R) mRNA expression was first detected in 9–12 mm granulosa tissue (Johnson et al. 1996). This LH sensitivity enables selected cells to produce progesterone in respond to LH (Asem & Hertelendy 1985). Treatment of F4/5 granulosa cells with LH stimulates LH-R mRNA expression; however, activin A prevented this induction (Davis et al. 2001), suggesting that activin A could act to regulate follicular maturation by preventing excessive or untimely LH-R expression. The reduction in ActRIIA (this study) and activin A (Lovell et al. 2003) suggests that the gradual reduction in activin activity could lead to increased LH-R expression as seen in F3–F1 granulosa cells (Johnson et al. 1996).

Inhibin A production increased progressively (≈100-fold increase from 5–6 mm to F1) as follicles progress through the preovulatory hierarchy (Lovell et al. 1998, 2003). Various factors have been shown to modulate secretion of inhibin A and progesterone from cultured chicken F1–F3 granulosa cells including FSH, LH, TGFα, insulin-like growth factor-I and activin A (Onagbesan & Peddie 1995, Lovell et al. 2002a,b). Changes in plasma inhibin A mirror the F1 granulosa inhibin A content, peaking around the time of the LH surge (Lovell et al. 2000) and may be involved in endocrine regulation of pituitary gonadotroph function, through interaction with betaglycan and ActRIIA as described by Lovell et al. (2005). The fact that the transition from F2 to F1 coincides with a significant increase in granulosa betaglycan and ActRIIA expression, with no alteration in ActRI, suggests that the F1 granulosa itself may be a key target for inhibin action in an autoregulatory feedback manner.

Inhibin A from granulosa cells may also modulate thecal steroid production by antagonizing the action of activin. Changes in thecal expression of betaglycan appear to mirror previously reported changes in thecal androstenedione production with levels increasing from F4 to a peak in F2 before decreasing in the F1 follicles (Llewelyn 1981, Etches & Duke 1984, Marrone & Hertelendy 1985). In mammals, inhibins stimulate and activin inhibits LH-induced androgen production by thecal cells (Hillier et al. 1991a,b, Wrathall & Knight 1995). Indeed, Rombouts et al. (1996) reported that inhibin and activin also have this antagonistic effects on androstenedione production by primary ovarian cells derived from chicken embryos. It is therefore likely that inhibin acting through betaglycan/ActRII binding has a paracrine action on thecal androstenedione production.

In conclusion, taken together with previous reports documenting the spatio-temporal pattern of expression of inhibins, activins and follistatin in the hen ovary, the present finding of divergent expression of activin receptor subtypes and the putative inhibin co-receptor, betaglycan, reinforce the view that inhibin, acting through betaglycan and ActRIIA/B and activins acting through ActRI and ActRIIA/B may play key roles during folliculogenesis in the avian ovary.

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