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# Oocyte regulation of anti-Müllerian hormone expression in granulosa cells during ovarian follicle development in mice

Nicholas A. Salmon, Alan H. Handyside, and Ieuan M. Joyce*

*School of Biology, University of Leeds, Leeds LS2 9JT, UK*

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## Abstract

In the ovarian follicle, anti-Müllerian hormone (*Amh*) mRNA is expressed in granulosa cells from primary to preovulatory stages but becomes restricted to cumulus cells following antrum formation. Anti-Müllerian hormone regulates follicle development by attenuating the effects of follicle stimulating hormone on follicle growth and inhibiting primordial follicle recruitment. To examine the role of the oocyte in regulating granulosa cell *Amh* expression in the mouse, isolated oocytes and granulosa cells were co-cultured and *Amh* mRNA levels were analysed by real-time RT-PCR. Expression in freshly isolated granulosa cells increased with preantral follicle development but was low in the cumulus and virtually absent in the mural granulosa cells of preovulatory follicles. When preantral granulosa cells were co-cultured with oocytes from early preantral, late preantral or preovulatory follicles, and when oocytes from preovulatory follicles were co-cultured with cumulus granulosa cells, *Amh* expression was increased at least 2-fold compared with granulosa cells cultured alone. With oocytes from preantral but not preovulatory follicles, this was a short-range effect only observed with granulosa cells in close apposition to oocytes. We conclude that stage-specific oocyte regulation of *Amh* expression may play a role in intra- and inter-follicular coordination of follicle development.
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*Keywords:* Oocyte; Anti-Müllerian hormone; Granulosa cells; Follicle; Ovary; Mouse

## Introduction

Ovarian folliculogenesis involves the coordinated development of an oocyte within a somatic cell structure consisting of granulosa cells, and later theca cells. Following the recruitment of quiescent primordial follicles into the pool of growing preantral follicles, follicle development is characterised by proliferation and differentiation of granulosa cells. At the same time, the oocyte grows and acquires developmental competence. With appropriate endocrine support, this process ultimately results in the development of a preovulatory follicle comprising a fully grown, embryogenically competent oocyte and granulosa cells divided into two subpopulations—cumulus cells that closely surround the oocyte and mural granulosa cells.

*Amh*, a member of the TGF-β family of growth and differentiation factors, encodes anti-Müllerian hormone (AMH) also known as Müllerian inhibiting substance (MIS) and is expressed by granulosa cells in growing and preovulatory follicles. By participating in two critical selection points, AMH is of key importance during follicle development: it decreases the responsiveness of growing follicles to FSH and inhibits the recruitment of primordial follicles into the pool of growing follicles (Durlinger et al., 1999, 2001, 2002a).

Despite its importance, regulation of expression of *Amh* during follicle development is not well understood. However, in mouse antral follicles, higher *Amh* mRNA expression is seen in cumulus cells than in mural granulosa cells (Munsterberg and Lovell-Badge, 1991). Since LH/CG receptor (*Lhcgr*), KIT ligand (*Kitl*), urokinase plasminogen activator (*Plau*) and prostaglandin-endoperoxide synthase 2 (*Ptgs2*) are also differentially expressed in cumulus and mural granulosa cells in preovulatory follicles and this differential expression has been shown to be regulated by paracrine signals from the oocyte (Canipari et al., 1995; Eppig et al., 1997; Joyce et al., 1999, 2001), the pattern of expression of *Amh* may suggest that the oocyte is involved in the regulation of this gene during later stages of follicle development.

* Corresponding author.
*E-mail address:* i.m.joyce@leeds.ac.uk (I.M. Joyce).

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At earlier stages of follicle development, little is known about oocyte regulation of granulosa cell gene expression. However, there is evidence that oocyte regulation of *Lhcgr*, *Kitl* and *Ptgs2* in granulosa cells is dependent upon the stage of oocyte development (Eppig et al., 1997; Joyce et al., 1999, 2001), suggesting that the factors mediating oocyte–granulosa cell interaction may change as the oocyte develops. Furthermore, a recent study has demonstrated a role for the oocyte in determining the rate at which follicles develop, indicating that oocyte regulation of gene expression during these earlier stages of folliculogenesis could be of functional significance (Eppig et al., 2002). Given that *Amh* is expressed during preantral stages of follicle development, it is therefore of considerable interest to determine if granulosa cell expression of *Amh* is regulated by the oocyte during these earlier stages. If so, this would not only indicate that oocyte regulation of granulosa cell gene expression is active during extended periods of follicle development, but would also suggest that growing oocytes regulate follicle growth by decreasing the responsiveness of growing follicles to FSH and play a role in regulating inter-follicular control over the recruitment of primordial follicles.

This study examined the role of the oocyte in regulating granulosa cell *Amh* expression during follicle development in the mouse. Experiments used oocytes and granulosa cells in co-culture followed by real-time RT-PCR to quantify *Amh* mRNA levels in granulosa cells. The results indicate that *Amh* mRNA levels in the granulosa cells of early and late preantral follicles and cumulus cells of preovulatory follicles are up-regulated by the oocyte in a stage-specific fashion.

## Materials and methods

### *Isolation and culture of granulosa cells and oocytes*

All cultures containing granulosa cells and oocytes were undertaken in 384-well plates (Corning Incorporated, Corning, NY) using 15-μl Waymouth culture medium (Invitrogen Life Technologies, Inchinnan, UK), supplemented with 0.23 mM pyruvic acid (Sigma-Aldrich, Poole, UK), 50 mg/l streptomycin sulphate (Sigma-Aldrich), 75 mg/l penicillin G (Sigma-Aldrich), 3 mg/ml bovine serum albumin (Sigma-Aldrich), 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml selenium (Sigma-Aldrich). Cultures were maintained at 37°C in 5% $CO_2$, 5% $O_2$, 90% $N_2$ for 24 h.

Preantral granulosa–oocyte complexes (GOC) from prepubertal mice were collected at early and late preantral stages of development. Shortly after birth in mice a wave of follicle development is initiated, thereby facilitating the isolation of follicles at specific stages of development depending on the age of the mouse. Early preantral GOC corresponding to Type 3b/4 follicles using the Pedersen and Peters (1968) classification were isolated from the ovaries of 6-day-old $F_1$ mice (FVB/N × C57BL6). Late preantral GOC corresponding to Type 5 follicles using the Pedersen and Peters (1968) classification were isolated from the ovaries of 12-day-old $F_1$ mice. GOC were isolated using collagenase digestion as described previously (Eppig and O'Brien, 1996). After 24 h in culture, GOC typically retained a spherical appearance with the oocyte centrally located within this sphere and limited attachment of the GOC to the culture plate. To obtain clumps of granulosa cells in the absence of oocytes, the GOC were oocytectomised using a fine-bored glass micropipette as described by Joyce et al. (1999). After 24 h in culture, individual granulosa cell clumps isolated in this way had organised into a near-spherical structure with limited attachment of the clumps to the culture plate. Denuded oocytes approximately 41 ± 0.9 μm were collected from early preantral follicles and denuded oocytes approximately 56 ± 1.25 μm were collected from late preantral follicles using calcium and magnesium free phosphate-buffered saline as described previously (Eppig et al., 1997).

Cumulus cell–oocyte complexes (COC) from preovulatory follicles (Type 8) were collected by needling the ovaries of 22-day-old mice that had been injected 44–48 h previously with 5 IU equine chorionic gonadotrophin (eCG). Experiments in which oocytes from preovulatory follicles were used were additionally supplemented with 10 μM milrinone (Sigma-Aldrich) to prevent spontaneous germinal vesicle breakdown. Milrinone is an inhibitor of the oocyte-specific phosphodiesterase-3 (Tsafriri et al., 1996). In preliminary experiments, this concentration of milrinone did not affect *Amh* mRNA expression by granulosa cells. Fully grown oocytes approximately 77 ± 0.76 μm in diameter were collected by gentle pipetting of COC and clumps of cumulus cells were obtained using a fine-bored glass micropipette to oocytectomise the COC.

Following the culture period, granulosa cells were separated from co-cultured oocytes and were snap frozen and stored for later analysis at −80°C.

### *Quantification of Amh mRNA levels using real-time RT-PCR*

Steady-state granulosa cell *Amh* mRNA levels were quantified relative to steady-state ribosomal protein L19 (*Rpl19*) mRNA levels using real-time PCR following reverse transcription of total RNA. In preliminary studies, *Rpl19* mRNA levels in granulosa cells did not differ following co-culture with oocytes. *Rpl19* mRNA levels have been widely employed in studies using relative quantification of granulosa cell gene expression levels (Eppig et al., 1997; Joyce et al., 1999, 2001; Otsuka and Shimasaki, 2002).

Total RNA was extracted from the granulosa cells using a total RNA extraction kit (RNeasy; Qiagen Ltd, Crawley, UK) as instructed in the manufacturer's handbook. An additional "on column" DNase step was used to reduce genomic DNA in the total RNA preparation below detectable levels. Reverse transcription of polyA RNA was achieved using Sensiscript reverse transcriptase (Qiagen Ltd), with the reaction primed using 5 mM oligo dT. RNase inhibitor (RNasin; Promega, Southampton, UK) was included in the

mixture to prevent premature degradation of the RNA. Complementary DNA was concentrated by addition of 2 ng glycogen, 1/10 volume of 3 M sodium acetate ($C_2H_3O_2Na$) pH 5.2, and 3 volumes of 100% ethanol (EtOH) followed by storage at −80°C for 1 h. The mixture was then spun at 13,000 rpm for 15 min at 4°C. The supernatant was removed and the pellet washed by the addition of 400 μl of 70% EtOH, it was then vortexed for 1 min and spun at 13,000 rpm for 15 min at 4°C. The supernatant was removed and the pellet resuspended in 20 μl of water.

Complementary DNA quantification was performed on a LightCycler using the LightCycler-Faststart DNA Master SYBR Green I Kit (Roche Molecular Biochemicals, Mannheim, Germany). The primer sequences for *Amh* were: forward, 5-GCAGTTGCTAGTCCTACATC-3 and reverse 5-TCATCCGCGTGAAACA GCG-3. The primer sequences for *Rpl19* were: forward 5-TGCCTCTAGTGT CCTCCGC-3 and reverse 5-ATCCGAGCATTGGCAGTACC. Reactions were performed in 10 μl, with the final reaction mix containing 6.2 μl water, 0.8 μl $MgCl_2$ (3 mM), 0.5 μl forward primer (500 nM), 0.5 μl reverse primer (500 nM), 1 μl LightCycler mix and 1 μl of cDNA template. The PCR protocol used an initial denaturing step at 95°C for 10 min followed by 45 cycles of 95°C for 10 s, 57°C for 6 s; 72°C for 15 s and 85°C for 2 s. Fluorescence was measured at the end of each cycle during the 85°C step. The final step was the generation of a melting curve in which the temperature was raised from 65°C to 95°C at 0.1°C/s, with the fluorescence being constantly measured, followed by a cooling stage of 40°C for 30 s. PCR reaction efficiency for each set of primers was measured in every run by including in the assay a serial dilution of granulosa cell cDNA prepared alongside cDNA from the experiment being analysed. *Amh* and *Rpl19* cDNA levels in this serial dilution spanned those in the samples. Crossing points (i.e. the cycle number at which the reaction begins the log linear phase of amplification) from the serial dilution were used to produce a slope that was then converted into PCR efficiency ($E$) using the equation $E = 10^{(-1/\text{slope})}$ (Pfaffl, 2001). All readings were taken using the "Fit Points Method" in which crossing points are measured at a constant fluorescence level.

### *Relative quantification of steady-state Amh mRNA and statistical analysis*

Relative quantification was performed using the equation:

$$\text{ratio} = \frac{(E^{\text{RPL}})^{\text{CP}}}{(E^{\text{AMH}})^{\text{CP}}}$$

where, $E^{\text{RPL}}$ is the efficiency of amplification of the reference gene *Rpl19*, $E^{\text{AMH}}$ is the efficiency of amplification of the target gene *Amh* and CP is the crossing point. Since crossing point and cDNA quantity are inversely related, the ratio obtained from the second equation is equal to the ratio of *Amh* cDNA/*Rpl19* cDNA.

Each experiment was replicated between three and six times and analysed using one-way ANOVA. When a significant effect among treatment groups was detected, the individual groups were compared with a Student–Newman–Keuls multiple-range post hoc test.

## Results

### *Amh mRNA expression through follicle development*

*Amh* mRNA expression was examined in freshly isolated granulosa cell populations from follicles at different stages of development (Fig. 1). *Amh* mRNA levels were lower in granulosa cells from the early preantral follicles of 6-day-old mice than in the late-preantral follicles of 12-day-old mice. In granulosa cells isolated from the preovulatory follicles of 22-day-old eCG-primed mice, *Amh* mRNA levels were lower than in granulosa cells from mid- or late-stage preantral follicles. However, within preovulatory follicles, higher *Amh* mRNA levels were seen in cumulus cells than in mural granulosa cells.

### *Oocyte regulation of Amh in late preantral follicles*

Oocyte regulation of *Amh* mRNA expression in granulosa cells from late preantral follicles was studied using

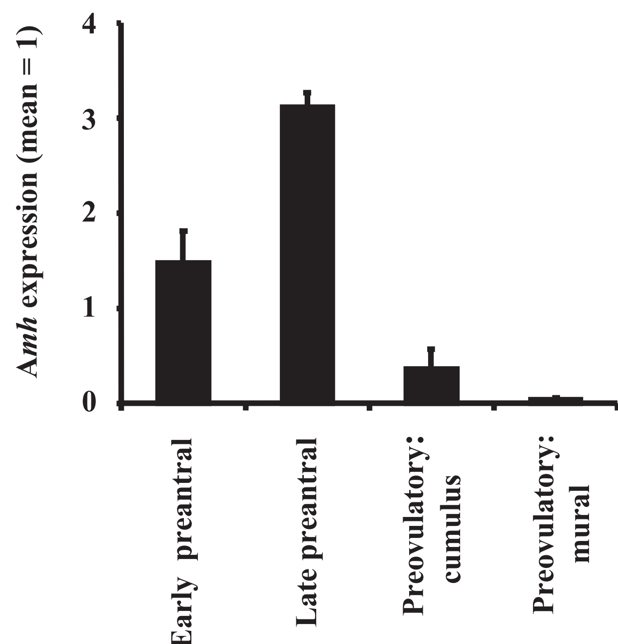



Fig. 1. *Amh* mRNA expression in granulosa cells from follicles at a range of developmental stages. Granulosa cells from early and late preantral follicles were isolated from the ovaries of 6- and 12-day-old mice, respectively. Cumulus and mural granulosa cells from preovulatory follicles were isolated from 22-day-old mice injected 44–48 h previously with 5IU eCG. Individual replicates composed of samples from each developmental stage were normalised so that the mean *Amh* mRNA levels were equal to 1. Data are presented as the mean ± SEM.

five treatment groups: intact late preantral GOC (two GOC per microliter of medium); late preantral granulosa cells cultured alone and late preantral granulosa cells co-cultured with two, four or eight late preantral follicle oocytes per microliter of medium (Fig. 2). After 24 h of culture, *Amh* mRNA levels in granulosa cells cultured alone were significantly lower than levels in granulosa cells in intact complexes ($P < 0.05$). Co-culture of eight oocytes per microliter with the granulosa cells resulted in *Amh* mRNA levels similar to those found in intact late GOC. *Amh* mRNA levels were intermediate in the groups containing two and four oocytes per microliter of medium.

*Short-range signals are required for oocyte regulation of Amh in late preantral follicles*

The importance of short-range effects on oocyte regulation of granulosa cell *Amh* mRNA expression in late preantral follicles was examined using late preantral granulosa cells either cultured alone or co-cultured with eight contemporaneous oocytes per microliter of medium. Following the 24 h culture period, granulosa cell clumps in the co-cultures were separated into two pools, those that had reestablished contact with oocytes and those that had not (Fig. 3a). *Amh* mRNA levels were significantly higher in granulosa cell clumps that had made contact with oocytes than in clumps that had either not established contact with oocytes or that had been cultured alone ($P < 0.05$; Fig. 3b). It is unlikely that this observation is an artefact of there being subpopulations of granulosa cells in the culture that were differentially 'selected' for reconnection with oocytes because (1) no morphological differences between the two pools of granulosa cells were apparent using light microscopy (see Fig. 3a); (2) both reconnected and unconnected granulosa cell clumps expressed similar levels of a range of genes normally expressed by granulosa cells including *Sf1* and *Gata4* (data not shown); (3) in cultures with larger numbers of co-cultured oocytes, proportionately more clumps of granulosa cells became reconnected (data not shown). Instead, selection for reconnection appears to have been a random event, dependent upon the proximity of oocytes and granulosa cell clumps at the start of culture.

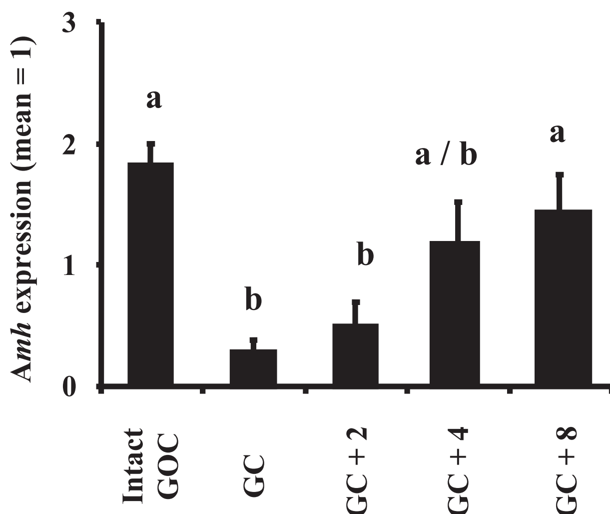
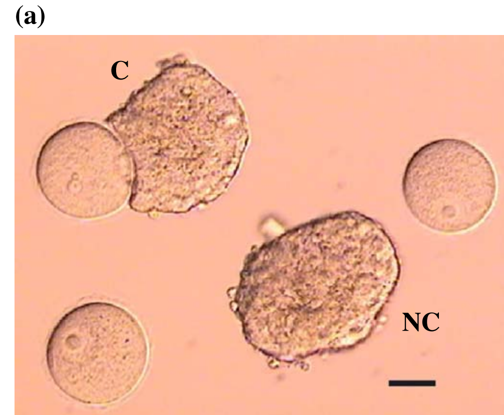



Fig. 2. The effect of late preantral follicle oocytes on *Amh* mRNA expression in late preantral granulosa–oocyte complexes (GOC). Both oocytes and preantral GOC were collected from 12-day-old mice. Preantral GOC were cultured intact, as granulosa cell clumps following oocytectomy (GC) or GC plus two, four or eight denuded oocytes per microliter of medium. Individual replicates were normalised so that the mean *Amh* mRNA levels were equal to 1. Data are presented as the mean ± SEM; bars with different letters are significantly different ($P < 0.05$).

(a)




(b)

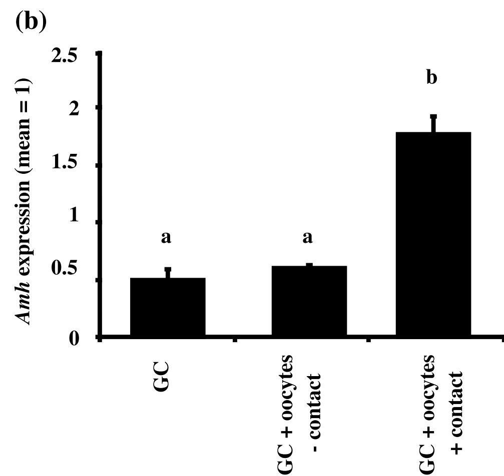



Fig. 3. (a) Photograph of oocytectomised late preantral granulosa–oocyte complexes and contemporaneous oocytes following a 24-h co-culture. Some granulosa cell clumps reestablished contact with the oocytes (C), whereas other granulosa cell clumps did not (NC). Scale bar represents 25 μm. (b) The effect of oocytes isolated from late preantral follicles on *Amh* mRNA expression in preantral granulosa–oocyte complexes (GOC). Both oocytes and preantral GOC were collected from 12-day-old mice. Preantral GOC were cultured as granulosa cell clumps following oocytectomy (GC) or GC plus eight denuded oocytes per microliter of medium; following 24 h in culture, granulosa cell clumps from the latter treatment were divided into those that had not reestablished contact with oocytes (−contact) and those that had (+contact). Individual replicates were normalised so that the mean *Amh* mRNA levels were equal to 1. Data are presented as the mean ± SEM; bars with different letters are significantly different ($P < 0.05$).

### *Oocytes from early preantral to preovulatory follicles up-regulate Amh expression*

The importance of the stage of development of the oocyte on *Amh* regulation was examined by determining the effect of oocytes from early preantral (Fig. 4) and preovulatory follicles (Fig. 5a) on *Amh* mRNA levels in granulosa cells isolated from late preantral follicles. To compensate for differences in oocyte volume, 20 early preantral follicle oocytes per microliter or three preovulatory follicle oocytes per microliter were used. At these concentrations, the total volume of oocytes in the cultures was approximately equivalent to eight late preantral follicle oocytes per microliter. Oocytes from early preantral follicles significantly increased *Amh* mRNA levels in vitro, but only when in contact with the granulosa cells ($P < 0.05$). In contrast, oocytes from preovulatory follicles significantly increased *Amh* mRNA levels in granulosa cells that had made contact with oocytes and in granulosa cells that had not made contact with oocytes ($P < 0.05$). To determine whether the concentration of oocyte-derived signalling factors was important in this effect, the number of oocytes co-cultured with granulosa cells was reduced to 0.5 oocytes per microliter of medium (Fig. 5b). With this number of preovulatory follicle oocytes, the effect on *Amh* mRNA expression was similar to that seen with oocytes from early and late preantral follicles, in that *Amh* mRNA levels were significantly elevated in granulosa cells in contact with oocytes, but not when there was no contact with oocytes.

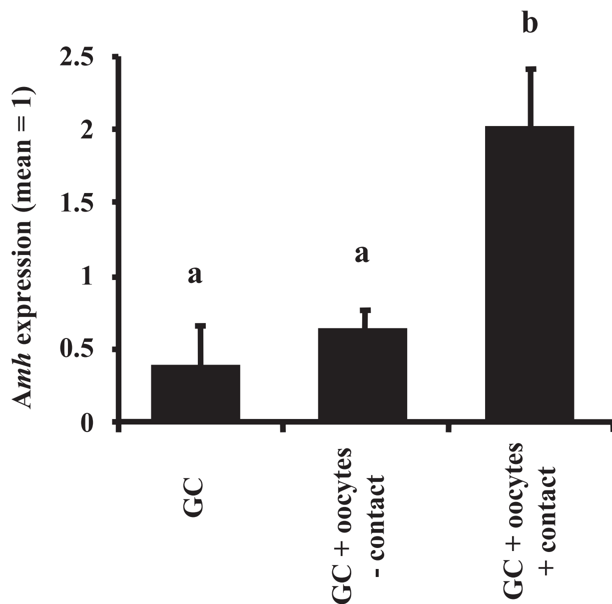



Fig. 4. The effect of oocytes isolated from early preantral follicles on *Amh* mRNA expression in granulosa cells from late preantral follicles. Oocytes and preantral GOC were collected from 6- and 12-day-old mice, respectively. Preantral GOC were cultured as granulosa cell clumps following oocytectomy (GC) or GC plus 20 denuded oocytes per microliter of medium; following 24 h in culture, granulosa cell clumps from the latter treatment were divided into those that had not reestablished contact with oocytes (−contact) and those that had (+contact). Individual replicates were normalised so that the mean *Amh* mRNA levels were equal to 1. Data are presented as the mean ± SEM; bars with different letters are significantly different ($P < 0.05$).

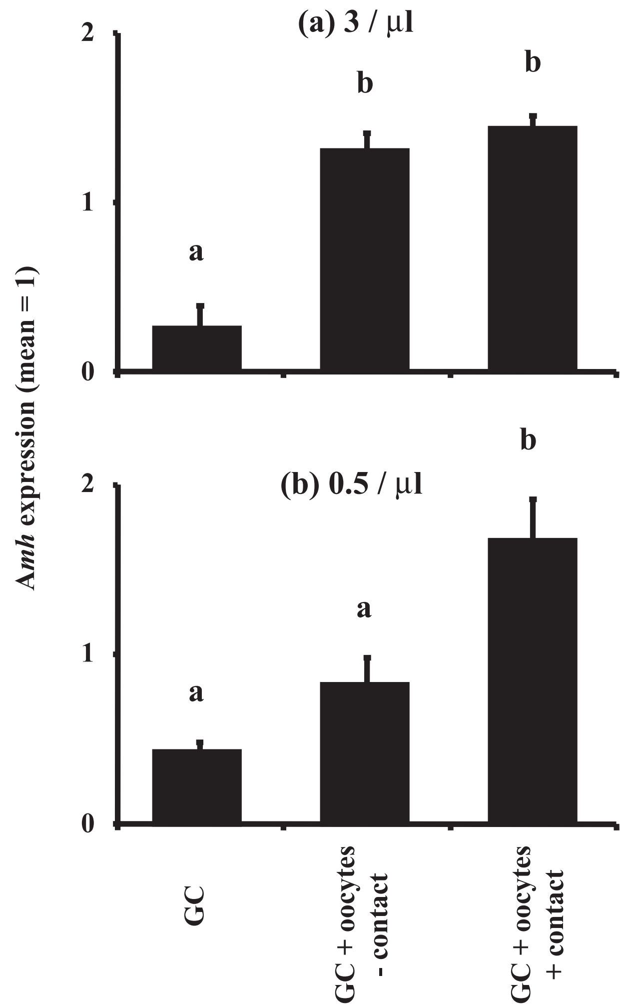



Fig. 5. The effect of fully grown oocytes on *Amh* mRNA expression in granulosa cells from late preantral follicles. Preantral GOC were cultured as granulosa cell clumps following oocytectomy (GC) or GC plus oocytes. Following 24 h in culture, granulosa cell clumps from the latter treatment were divided into those that had not reestablished contact with oocytes (−contact) and those that had (+contact). (a) The GC plus oocytes group contained three fully grown oocytes per microliter of medium (3/µl). (b) The GC plus oocytes group contained 0.5 fully grown oocytes per µl of medium (0.5/µl). Individual replicates were normalised so that the mean *Amh* mRNA levels were equal to 1. Data are presented as the mean ± SEM; bars with different letters are significantly different ($P < 0.05$).

### *Oocyte regulation of Amh expression in cumulus cells*

To determine whether oocytes from preovulatory follicles regulate *Amh* mRNA expression in the cumulus cells of preovulatory follicles, intact COC were cultured at a concentration of three per microliter of medium; cumulus cells were cultured alone; and cumulus cells were co-cultured

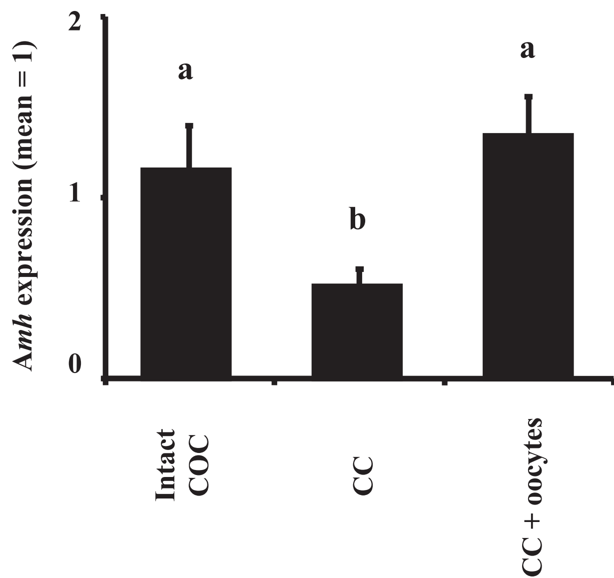



Fig. 6. The effect of fully grown oocytes on *Amh* mRNA expression in cumulus–oocyte complexes (COC). COC isolated from preovulatory follicles were cultured intact at a concentration of three per microliter, in clumps of cumulus cells following oocytectomy (CC), or as CC plus three denuded oocytes per microliter of medium. Individual replicates were normalised so that the mean *Amh* mRNA levels were equal to 1. Data are presented as the mean ± SEM; bars with different letters are significantly different ($P < 0.05$).

with three preovulatory follicle oocytes per microliter of medium (Fig. 6). In the presence of oocytes, either in intact COC or in co-culture, *Amh* expression was significantly higher than in cumulus cells cultured in the absence of oocytes ($P < 0.05$).

## Discussion

This study tested the hypothesis that the oocyte regulates granulosa cell *Amh* expression during preantral and preovulatory stages of follicle development. The results show that oocytes from early preantral, late preantral and preovulatory follicles up-regulate *Amh* mRNA levels in granulosa cells, in a fashion that is dependent upon the developmental stage of the oocyte. These findings therefore suggest that oocyte regulation of granulosa cell gene expression occurs during extended periods of follicle development and that oocyte regulation of *Amh* expression may play a role in intra- and inter-follicular coordination of follicle development.

The finding that oocytes from preantral and preovulatory follicles up-regulate granulosa cell expression of *Amh* is based on the observation that oocytectomy of both granulosa–oocyte complexes from late preantral follicles and cumulus–oocyte complexes from preovulatory follicles decreased granulosa cell *Amh* mRNA levels compared to complexes cultured with an intact oocyte. At the same time, co-culture of oocytes from late preantral and preovulatory follicles with their contemporaneous granulosa cells increased granulosa cell *Amh* mRNA levels, indicating that the effect of oocytectomy was due to the loss of signal(s) from the oocyte rather than an artefact of the oocytectomy procedure.

The up-regulation of *Amh* mRNA levels in granulosa cells of preantral follicles by oocytes from early and late preantral and preovulatory follicles demonstrated here indicate that oocyte regulation of granulosa cells occurs during these developmental stages. In *Gdf9*$^{null}$ mice, oocyte-derived signals are essential for the further growth of primary follicles (Dong et al., 1996). Oocytes have also been shown to regulate granulosa cell expression of the oocyte growth-promoting gene *Kitl* in early antral follicles, as well as *Ptgs2*, a gene required for appropriate cumulus expansion in ovulatory follicles (Joyce et al., 1999, 2001). Taken together, there is now evidence therefore for oocyte regulation of granulosa cells from primary through to ovulatory follicle stages, establishing the oocyte as a key regulator of follicle development.

Oocyte regulation of *Amh* during preantral follicle development is of functional interest for two reasons. Firstly, AMH attenuates FSH-stimulated growth of preantral and early antral follicles. Oocyte regulation of the expression of *Amh* could therefore act as a control point for regulating the development of growing follicles, thereby providing an explanation to the problem of understanding how oocytes determine the rate at which follicles develop (Eppig et al., 2002). Given that AMH attenuates FSH-stimulated follicle growth, it seems likely that further studies will identify oocyte-regulated granulosa cell expressed genes that increase the rate of follicle development. Secondly, AMH inhibits primordial follicle recruitment. It has been proposed that this occurs in an inter-follicular fashion, whereby AMH production from growing follicles signals to primordial follicles to inhibit recruitment (Durlinger et al., 2002b). Therefore, oocyte up-regulation of the production of AMH by growing follicles would be expected to lead to a reduced number of primordial follicles recruited into the growing pool. This being the case, it is possible that a negative feedback mechanism is established, with the rate of recruitment from the pool of primordial follicles effectively determined by the number of oocytes in the pool of growing follicles.

By the preovulatory follicle stage, oocyte control over granulosa cell *Amh* mRNA levels appears to decline since there is a considerable reduction in *Amh* levels in comparison with those found in preantral follicles. The reasons for this are not known but may, for example, be related to changes in sensitivity to endocrine stimulation. Preliminary studies examining the effect of recombinant FSH on *Amh* mRNA expression in granulosa cells in culture have been inconclusive (data not shown). However, the observation that *Amh* mRNA levels were higher in cumulus cells than in mural granulosa cells, coupled with the data showing oocytes from preovulatory follicles up-regulate

granulosa cell *Amh* mRNA expression in vitro, indicates that the oocyte continues to exert an up-regulatory stimulus on *Amh* mRNA expression during preovulatory follicle development.

Oocytes at all stages examined up-regulated granulosa cell *Amh* expression. However, evidence of stage specificity was provided by the observation that oocyte regulation of granulosa cell *Amh* expression was dependent on short-range effects for oocytes isolated from preantral follicles, but with oocytes from preovulatory follicles, this dependence was only observed at low numbers of oocytes. Whether this short-range effect is in fact contact-dependent or results from low-level production of a highly labile secreted factor is unclear. However, because contact was not necessary for the *Amh* regulatory effect of oocytes from preovulatory follicles, at this later stage of development a secreted factor is involved. Such stage-specific differences may be functionally significant. In preantral follicles, the dependence on short-range effects would ensure that the direct sphere of influence of oocyte-derived signals is limited to granulosa cells at a stage when the number of granulosa cells is low. In preovulatory follicles, on the other hand, the existence of a strong, secreted signal from the oocyte may be important in enabling the oocyte to promote appropriate patterns of granulosa cell gene expression within a follicular structure composed of many more cells as well as a large fluid-filled antrum.

Developmental changes in the way oocytes regulate granulosa cells have also been found in other studies. For example, growing oocytes from late preantral follicles up-regulate granulosa cell expression of *Kitl*, whereas fully grown oocytes down-regulate expression of this gene (Joyce et al., 1999). Furthermore, fully grown but not growing oocytes from late preantral follicles promote cumulus cell expression of *Ptgs2*, whereas growing oocytes from late preantral follicles have only partial ability to down-regulate mural granulosa cell expression of *Lhcgr* mRNA in comparison with fully grown oocytes (Eppig et al., 1997; Joyce et al., 2001). These results, coupled with those from the current study, suggest that there may be a high level of complexity in the developmental dynamics of oocyte regulation of granulosa cells. Indeed, an array of oocyte-derived factors, including GDF-9, BMP-15, OOSP-1, FGF-8 and BMP-6 (Dong et al., 1996; Elvin et al., 1999; Otsuka et al., 2001; Valve et al., 1997; Yan et al., 2001a,b) have been identified that may underlie such complexity. The identity of the specific factor(s) involved in oocyte regulation of granulosa cell *Amh* expression are currently being investigated.

This study used prepubertal mice to facilitate the isolation of follicles at defined stages of development. Follicles from prepubertal mice are fully functional when such animals are stimulated with exogenous gonadotrophin hormones during the latter, preovulatory, stages of follicle development. Given that expression of *Amh* by granulosa cells is dependent on the stage of follicle development and not on the age of the mouse (Munsterberg and Lovell-Badge, 1991), it seems likely that oocyte regulation of *Amh* expression in granulosa cells occurs in a similar fashion in both prepubertal and adult animals.

The studies reported here provide experimental evidence that oocytes from early preantral, late preantral and preovulatory follicles are involved in the regulation of expression of *Amh* mRNA in granulosa cells, in a fashion that is dependent upon the developmental stage of the oocyte. These findings indicate that the oocyte regulates granulosa cell gene expression during extended periods of follicle development. Furthermore, the results suggest that the oocyte plays a role in intra-follicular regulation of the rate of follicle growth as well as in inter-follicular inhibition of primordial follicle recruitment.

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