Association of luteinizing hormone receptor gene expression with cell cycle progression in granulosa cells

Jennifer D. Cannon, Srinivas V. Seekallu, Catherine A. VandeVoort, and Charles L. Chaffin

Association of luteinizing hormone receptor gene expression with cell cycle progression in granulosa cells. Am J Physiol Endocrinol Metab 296: E1392–E1399, 2009. First published March 17, 2009; doi:10.1152/ajpendo.90965.2008.—During hormonally induced ovarian follicle growth, granulosa cell proliferation increases and returns to baseline prior to the administration of an ovulatory stimulus. Several key genes appear to follow a similar pattern, including the luteinizing hormone receptor (LHCGR), suggesting an association between cell cycle progression and gene expression. The expression of LHCGR mRNA in granulosa cells isolated from immature rats and treated in culture with FSH increased in a time-dependent manner, whereas administration of the cell cycle inhibitor mimosine completely suppressed expression. Although forskolin was able to induce luteinization in cells treated with mimosine, human chorionic gonadotropin had no effect, indicating the functional loss of LHCGR. The effects of mimosine on cell cycle progression and LHCGR mRNA expression were reversible within 24 h of mimosine removal. Cell cycle inhibition did not alter the stability of LHCGR mRNA, indicating that the primary effect was at the transcriptional level. To determine whether the relationship between LHCGR expression and cell cycle were relevant in vivo, immature rats were given a bolus of PMSG, followed by a second injection of either saline or PMSG 24 h later to augment levels of proliferation. The expression of LHCGR mRNA was elevated in the ovaries of animals receiving a supplement of PMSG. Mimosine also blocked cell cycle progression and LHCGR mRNA expression in macaque granulosa cells isolated following controlled ovarian stimulation cycles and in two different mouse Leydig tumor lines. These data collectively indicate that LHCGR mRNA is expressed as a function of the passage of cells across the G1-S phase boundary.

granulosa cell; gene expression; ovary; proliferation

THE GROWTH AND DIFFERENTIATION of the ovarian follicle from small antral to preovulatory involves an increase in the expression of several genes key to follicle development, including follicle-stimulating hormone receptor (FSHR), luteinizing hormone receptor (LHCGR), and aromatase (cyp19A1). Granulosa cell proliferation during antral follicle growth in immature rats peaks 24–42 h after stimulation with pregnant mare’s serum gonadotropin (PMSG) and returns to baseline (pre-PMSG) levels at 48 h [prior to the administration of an ovulatory human chorionic gonadotropin (hCG) bolus] (3, 13, 20). During this time, the expression of LHCGR mRNA increases in a time-dependent manner until 42 h, after which expression levels plateau between 42–48 h post-PMSG, suggesting that cell cycle progression and LHCGR expression may be mechanistically linked.

Although deletion of the cyclin D2 (ccnd2) gene renders mice infertile, the absence of its cyclin-dependent kinase (cdk) partner cdk4 does not prevent follicle growth (21, 27). In contrast, the expression and activity of cyclin E (CCNE)/CDK2 is the hormonally regulated step in cell cycle progression during antral follicle growth and associates closely with the percentage of granulosa cells in S phase (3). Although CDK2 activity promotes cell cycle progression through phosphorylation of retinoblastoma and the subsequent release of CDK2 activity promotes cell cycle progression through phosphorylation of retinoblastoma and the subsequent release of E2F transcription factors, a number of other CDK2 protein targets have been identified, including the transcription factors Sp1, p53, MEF, and CBP/p300 (1, 12, 17, 22).

The activity of CDK2 can be inhibited pharmacologically; for example, mimosine is an amino acid that can reversibly block mammalian cells late in the first gap phase (G1) of the cell cycle (28). Treatment of HeLa cells with mimosine inhibits global protein synthesis by 50%, although some genes such as the cyclin-dependent kinase inhibitor p27 and differentiation-related gene 1 (Drg 1) are increased, suggesting that mimosine is not a general protein synthesis inhibitor (7, 8). Mimosine has also been used to synchronize porcine granulosa cells in G1 with a recovery of cell cycle progression following withdrawal of mimosine (28).

The goals of the present study were to evaluate the association between transit of granulosa cells from the first gap phase (G1) into the DNA synthesis (S) phase of the cell cycle and levels of LHCGR mRNA and to determine whether transit of granulosa cells across the G1-S boundary altered the stability of the LHCGR transcript.

MATERIALS AND METHODS

Reagents. FSH, Long R3 (LR)-IGF-I, activin-A, insulin-transferrin-selenium (ITS), and propidium iodide were obtained from Sigma Chemical (St. Louis, MO). 1-Mimosine was from Calbiochem (San Diego, CA) and olomoucine was from Tocris (Ellisville, MO). DMEM/F12 culture medium was from Life Technologies (Rockville, MD) and RPMI 1640 was from ATCC (Manassas, VA). Penicillin-streptomycin and gentamycin were from GIBCO (Carlsbad, CA). [3H]Thymidine was from MP Biomedicals (Irvine, CA).

Rat granulosa cell culture. All animal procedures were approved by the University of Maryland Animal Care and Use Committee and were in accordance with the National Institutes of Health Guide to the Care and Use of Laboratory Animals. Immature Sprague-Dawley rats (Harlan, Madison, WI) were euthanized on postnatal days 26–27 by decapitation following CO2 anesthesia. Ovaries were harvested and crushed by using 25-gauge needles (3).
The ovaries were shredded rather than punctured because most follicles in unstimulated immature rats are preantral. The level of aromatase mRNA in shredded ovaries increases with time following PMSG, suggesting a high proportion of granulosa cells in the shredded cell population. The resulting granulosa-enriched cells were cultured in serum-coated 24-well plates in DMEM/F12 medium containing 1X penicillin-streptomycin, 10 μM testosterone, 25 ng/ml ovine FSH, 100 ng/ml LR-IGF-I, and 25 ng/ml activin A at 37 °C in 95% air-5% CO2. After 24 h of culture, media were refreshed and either the cell cycle inhibitor L-mimosine (1 mM) or olomoucine (200 μM) or their respective vehicles (10% NaHCO3 or ethanol, respectively) were added to the cells. The cultures proceeded for an additional 24 h before being harvested, or in some cases media being refreshed with the addition of hCG (10 IU/ml), forskolin (10 μM), or control for 6 h to examine events during luteinization in vitro. Cells were either lysed in RNAqueous-Micro lysis buffer (Ambion/Applied Biosystems, Austin, TX) and frozen for later RNA isolation, trypsinized and prepared for flow cytometry, or processed for [3H]thymidine incorporation or harvest of RNA.

Flow cytometry. The percentage of cells in S phase of the cell cycle was determined by flow cytometry (19). Cells were briefly trypsinized and washed (400 g for 10 min at 4°C) two times in ice-cold fluorescence-activated cell-sorting (FACS) sample buffer (0.1% glucose-PBS) and resuspended in 100–200 μl of FACS sample buffer to obtain a single-cell suspension. Cells were fixed by dropwise addition of 1 ml ice-cold 70% ethanol whereas vortexing. Ethanol-fixed cells were stored at 4°C for at least 24 h before propidium iodide (PI) staining. Cells were centrifuged with all but 100–200 μl ethanol removed and then treated with 1 ml of PI staining solution (0.1 mg/ml PI, 0.5 mg/ml RNaS A in FACS sample buffer). Stained cells were held at room temperature for at least 1 h before FACS analysis. Immediately before analysis, cells were passed through a Falcon 35 μM nylon mesh cell strainer cap (BD Biosciences, Bedford, MA) to remove aggregated cells. Flow cytometric measurements of forward scatter, side scatter, and PI fluorescence were made by using a three-color FACSscan flow cytometer (Becton Dickinson, San Jose, CA) to determine DNA content. Cells were chosen for DNA content analysis according to the PI pulse area parameter (FL2-A). Data acquisition was performed with CellQuest (version 3.3) software and data analysis with ModFit LT for Macintosh (version 3.2) software (Verity Software House, Topsham, ME).

RESULTS

By shredding ovaries from immature rats, a granulosa cell-enriched population was isolated that expresses high levels of aromatase mRNA expression (3). The culture of these cells for 48 h in the presence of FSH + IGF-I did not promote movement into S phase whereas the addition of activin A

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significantly ($P < 0.05$) increased $[^{3}]$H$/$thymidine incorporation by 24 h and again at 48 h (Fig. 1A). Similarly, LHCGR mRNA increased 24 and 48 h after the onset of culture in the presence of FSH + LR-IGF-I + activin A (Fig. 1B).

To test the effects of cell cycle arrest on LHCGR mRNA, cells were cultured for 24 h in the presence of activin A, and mimosine or vehicle control was subsequently added and $[^{3}]$H$/$thymidine incorporation determined 24 h later. Mimosine markedly reduced $[^{3}]$H$/$thymidine incorporation (Fig. 2A) and also completely inhibited the expression of LHCGR mRNA (Fig. 2B), although mimosine did not alter viability (Fig. 2C).

![Fig. 1. Associated changes in $[^{3}]$H$/$thymidine incorporation and luteinizing hormone receptor (LHCGR) mRNA during granulosa cell differentiation in vitro. A granulosa cell-enriched population of cells obtained from immature rat ovaries was cultured for up to 48 h in the presence of 10 $\mu$M testosterone, 25 ng/ml ovine FSH, and 100 ng/ml LR-IGF-I with or without 25 ng/ml activin A. A: transit of cells into S phase was determined by $[^{3}]$H$/$thymidine incorporation. B: expression of LHCGR mRNA was assessed by real-time RT-PCR and data were normalized to the internal standard RPL32. Different superscript letters denote significant differences between times and treatments (2-way ANOVA with 1 repeated measure; $n = 4$ animals).](http://ajpendo.physiology.org/)

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As an in vitro bioassay of LH signaling, the expression of progesterone receptor (PR) mRNA was assessed before or after hCG or forskolin treatment. PR mRNA is an excellent gene marker of luteinization known to be dependent on activation of LHCGR (24). Cells were cultured for 48 h, the final 24 h in the presence of vehicle or mimosine, and PR mRNA was induced with either hCG or forskolin for 6 h. In vehicle-treated cells, hCG induced an 8.7-fold increase ($P < 0.05$) in PR mRNA over FSH alone, whereas forskolin increased PR mRNA 24-fold ($P < 0.05$). The expression of PR mRNA was not induced by hCG in cells treated with mimosine, although forskolin increased PR mRNA levels ($P < 0.05$) 21-fold relative to FSH only (Fig. 3). Basal

![Fig. 2. Pharmacological inhibition of cell cycle reduces LHCGR mRNA levels. Granulosa-enriched cells obtained from immature rats were cultured in the presence of 10 $\mu$M testosterone, 25 ng/ml ovine FSH, 100 ng/ml LR-IGF-I, and 25 ng/ml activin for 24 h. Media were changed to include 1 mM of the cell cycle inhibitor L-mimosine (mim) or vehicle control (ctrl; 10% NaHCO$_3$) for an additional 24 h. A: transit of cells into S phase was determined by $[^{3}]$H$/$thymidine incorporation. B: expression of LHCGR mRNA was assessed by real-time RT-PCR and data were normalized to the internal standard RPL32. C: media levels of formazan (an indicator of cell viability) were measured as described in MATERIALS AND METHODS. *Significant differences compared with control cultures (t-test, repeated measures; $n = 4$ animals).](http://ajpendo.physiology.org/)

**Table 1. Probe and primers sequences (5'-3') for real-time RT-PCR**

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<thead>
<tr>
<th>Forward Primer</th>
<th>Probe</th>
<th>Reverse Primer</th>
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<tr>
<td>LHCGR</td>
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<td>TCTCGGCTGCTTTGAAAGTGC</td>
</tr>
<tr>
<td>RPL19</td>
<td>CCGGAATGACCAAATGAAAAGC</td>
<td>ATGCCAATCCTGGATGAGAATCC</td>
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levels of PR mRNA (FSH only) were reduced significantly ($P < 0.05$) by mimosine treatment.

The reversibility of mimosine-induced suppression of cell cycle and LHCGR mRNA was tested by treating cells with activin A and mimosine for 24 h. Cell cycle inhibition was reversed 24 h after removal of mimosine from cultures, the same time at which LHCGR mRNA increased (Fig. 4, A and B). Pooling all the time points together, a significant ($P < 0.05$) positive correlation ($r = 0.6$) exists between [3H]thymidine incorporation and LHCGR mRNA expression (Fig. 4 C).

Preliminary experiments indicated that mimosine led to a ~50% reduction in LHCGR mRNA within 4 h (data not presented). To test the hypothesis that cell cycle arrest increased LHCGR mRNA degradation, cells were cultured for 24 h in the presence of activin A and then treated with vehicle or mimosine for 4 h, followed by up to 3 h of vehicle or mimosine with actinomycin D. The slope of LHCGR mRNA decay was not different for vehicle and mimosine treatments (Fig. 5).

The association of cell cycle progression with LHCGR mRNA in vivo was tested by PMSG stimulation of immature rats. Administration of PMSG to immature rats increased the percentage of cells in S phase 12 h postinjection, and these levels returned to baseline (0 h) at 48 h (Fig. 6 A; Ref. 3). A second injection of PMSG at 24 h prevented the 48-h reduction in S phase relative to saline controls ($P < 0.05$). The expression of LHCGR mRNA increased fivefold ($P < 0.05$) 24 h after the initial injection of PMSG and did not change significantly at 48 h in animals receiving a secondary injection of saline. In contrast, administration of a second injection of PMSG at 24 h resulted in a significant ($P < 0.05$) increase in LHCGR mRNA at 48 h (Fig. 6 B). The qualitative amount of follicular atresia as determined by TUNEL stain was not different between the PMSG/PMSG and PMSG/saline groups (data not presented).

The observation that LHCGR mRNA expression is associated with transit into S phase was extended to several other LH/CG responsive cells. Macaque nuluteinized granulosa cells aspirated prior to an ovulatory stimulus were cultured in the presence FSH + ITS with or without mimosine for 24 h. Mimosine reduced ($P < 0.05$) [3H]thyidine incorporation by approximately ninefold whereas LHCGR mRNA was similarly reduced ($P < 0.05$) (Fig. 7). This effect was further established in two Leydig tumor cells lines, mLTC-1 and MA-10 cells; in both cases, mimosine reduced [3H]thyidine incorporation and LHCGR mRNA expression (Fig. 7).

**Fig. 3.** The reduction in LHCGR mRNA corresponds with a lack responsiveness to human chorionic gonadotropin (hCG). Granulosa-enriched cells obtained from immature rats were cultured in the presence of 10 μM testosterone, 25 ng/ml ovine FSH, 100 ng/ml LR-IGF-I, and 25 ng/ml activin for 24 h. Media were changed to include 1 mM of the cell cycle inhibitor L-mimosine or vehicle control (10% NaHCO₃) for an additional 24 h. Media were refreshed with or without hCG (8 IU/ml) or forskolin (Fo; 10 μM) for 6 h. The expression of progesterone receptor (PR) mRNA was assessed by real-time RT-PCR and data were normalized to the internal standard RPL32. Different superscript letters denote significant differences between times and treatments (2-way ANOVA with 1 repeated measure; $n = 4$ animals).

**Fig. 4.** Mimosine-mediated suppression of LHCGR mRNA is reversible. Granulosa-enriched cells obtained from immature rats were cultured in the presence of 10 μM testosterone, 25 ng/ml ovine FSH, 100 ng/ml LR-IGF-I, and 25 ng/ml activin for 24 h with 1 mM mimosine. Cultures were rinsed and subsequently cultured in media without mimosine for up to 48 h. A: transit of cells into S phase was determined by [3H]thymidine incorporation. $A$: expression of LHCGR mRNA was assessed by real-time RT-PCR and data were normalized to the internal standard RPL32. C: [3H]thymidine incorporation and LHCGR mRNA expression from all samples across time were positively correlated. *Significant differences compared with 0 h (prior to reversal of cell cycle inhibition) (1-way ANOVA, 1 repeated measure; $n = 4$ animals).
Because it is difficult to ensure the specificity of mimosine, a second G1-arresting compound, olomoucine, was evaluated. Treatment of rat granulosa-enriched cells with olomoucine for 24 h did not significantly alter viability (data not presented), whereas $[^3]$Hthymidine incorporation was decreased by $\approx 5$-fold and LHCGR mRNA reduced by 18-fold ($P < 0.05$) (Fig. 8).

**DISCUSSION**

The goal of this study was to test the hypothesis that the expression of LHCGR mRNA was strongly associated with the transit of granulosa cells across the G1-S phase boundary. Pharmacologically inhibiting cell cycle progression reduced the expression of LHCGR mRNA in preovulatory granulosa cells to nearly undetectable levels, an effect that prevented these cells from responding to hCG. These observations were extended to intact animals, where higher levels of proliferation in ovary associate with increased expression of LHCGR mRNA. Importantly, the relationship between LHCGR expres-
Although proliferation of granulosa cells has also been suggested to play an important role in maturation to preovulatory status, recent data indicate that granulosa cell proliferation ceases near the time that LHCGR mRNA expression reaches its peak but well before an ovulatory stimulus (3). Cannon et al. (3) showed that, between 36–42 h after PMSG stimulation of immature rats, the proportion of granulosa cells in S phase was at the maximum level and during that 6 h interval, LHCGR mRNA increased by 60%; however, between 42–48 h after PMSG, the proportion of cells in S phase declined back to baseline whereas LHCGR mRNA increased less than 3%. These data suggest that a regulatory relationship exists between cell cycle progression and the expression of the key gene leading to the final maturation of the follicle to preovulatory status. Importantly, these changes are specific to mRNA, not necessarily to protein. It is possible that attenuating LH receptor expression in late preovulatory follicles prevents premature luteinization or is involved in LHCGR mRNA downregulation after an ovulatory stimulus (16).

A culture model was established in which ovaries from immature rat ovaries was cultivated for up to 48 h in the presence of 10 μM testosterone, 25 ng/ml ovine FSH, 100 ng/ml LR-IGF-I with or without 25 ng/ml activin A with vehicle control (ethanol) or 200 μM olomoucine. A granulosa cell-enriched population (3). These cells do not respond to FSH stimulation alone but require the addition of activin A to promote cell cycle progression and the coordinate increase in LHCGR mRNA. This temporal profile closely resembles in vivo stimulation, in which the proportion of cells in S phase and LHCGR mRNA both increase between 6–12 h following PMSG. The vast majority of cells entering S phase in the ovary following PMSG are in the granulosa cell layer (2), and thus it is likely that the [3H]thymidine incorporation and changes in LHCGR mRNA expression occur in the granulosa cell fraction of the cell cultures.

By blocking the progression of granulosa cells across the G1-S phase boundary, the expression of LHCGR mRNA is almost completely lost. Because LHCGR mRNA exists in vivo as multiple splice variants (9), a bioassay of LH action was used to verify the loss of LHCGR signaling. The expression of PR mRNA was used as a gene marker of LH receptor activity; this gene is robustly increased by LH or hCG both in vivo and in vitro in multiple species (5, 24) and thus serves as a sensitive and reliable indicator of LH receptor stimulation. PR mRNA increased in response to hCG or forskolin in cultures treated with vehicle; however, blockade of cell cycle progression with mimosine completely eliminated the ability of cells to respond to hCG, although forskolin was still able to promote PR expression. This indicates that the cells retained the general ability to respond to PKA-inducing stimuli, although it is clear that the LH receptor is either not expressed and/or not functional in cells treated with mimosine. The apparent blunting of forskolin effects following mimosine treatment are due almost entirely to reduced basal levels of PR mRNA rather than the inability of forskolin to induce PR mRNA; in vehicle-treated cells, PR mRNA increased 24-fold in response to forskolin and 21-fold in mimosine-treated cultures. Thus both LHCGR mRNA and LH receptor bioactivity are markedly suppressed in granulosa cells when cell cycle is arrested in late G1.

Many pharmacological inhibitors of the cell cycle are reversible, including mimosine. This property was utilized to further verify that cells arrested in G1 with attendant reduction in LHCGR mRNA levels could reenter the cell cycle and increase LHCGR expression. Following removal of mimosine from culture, it takes between 12 and 24 h to reestablish cell cycle progression (28) and LHCGR expression. A strong positive correlation was established between LHCGR and [3H]thymidine incorporation, supporting the hypothesis that cell cycle progression regulates LHCGR mRNA levels. It has recently been demonstrated that the stability of LHCGR mRNA is a critical determinant in expression (15, 23) and thus, on the basis of the rapidity of change and the tight association between S phase and gene expression, it was hypothesized that cell cycle arrest led to destabilization of the LHCGR mRNA. Interestingly, no evidence was found in the present study that cell cycle arrest promoted message destabilization, suggesting instead that the rate of transcription is altered by the passage of cells into S phase. The difference in LHCGR mRNA levels prior to actinomycin D addition reflects the pretreatment with vehicle or mimosine; a short exposure to mimosine (4 h) was chosen to reduce cell cycle progression as much as possible without completely losing the ability to detect LHCGR mRNA (cf. Fig. 2C). The incorporation of [3H]thymidine was reduced by 67-fold after only 4 h of mimosine treatment (data not presented), whereas LHCGR mRNA levels...
were only reduced by twofold. This additionally suggests that cell cycle arrest precedes the reduction in LHCGR mRNA, supporting a mechanistic link between the cell cycle and gene expression.

The modulation of the cell cycle in ovarian cells in vivo has proven difficult (14). For example, direct injection of the cell cycle inhibitor olomoucine under the bursa of exteriorized ovaries results in abnormal follicular morphology within 24 h (data not presented). Granulosa cells in the latter stages of follicle growth (36–48 h post-PMSG) are largely arrested in G1 phase of the cell cycle (3, 13). By administering a second injection of PMSG at 24 h, the proportion of cells in S phase could be maintained or augmented. Importantly, it is possible to achieve the same effect by using recombinant human FSH, and thus the effects on cell cycle are most likely due to the FSH receptor stimulatory effects of PMSG rather than LH receptor agonism. In this model, the levels of LHCGR mRNA do not increase significantly between 24–48 h post-PMSG. However, by increasing the percentage of cells in S phase even modestly with a second injection of PMSG, LHCGR mRNA was increased significantly vs. 24- and 48-h single PMSG-injected animals. Importantly, histology was not grossly affected by a second injection of PMSG, and was there any evidence for increased apoptosis relative to the saline injected control group. Thus LHCGR mRNA expression is associated with cell cycle progression in vivo.

The relationship between cell cycle progression and LHCGR mRNA expression was extended to three additional cell types: macaque granulosa cells isolated during a hormonal stimulation protocol prior to an ovulatory stimulus, and two murine macrophage and LHCGR mRNA expression is associated with cell cycle progression.

Although it is not clear what leads to the reduction in cell cycle progression in preovulatory follicles, a feedback loop may exist in the follicle, in which early antral follicle growth is driven in part by cell cycle progression and associated gene expression, whereas later maturation of the follicle is slowed as a consequence of cell cycle arrest.

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REFERENCES


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Mimosine induced a nearly complete attenuation of [3H]thy-FSH promoted in macaque granulosa cells with the addition of the hormone in a step in granulosa cell proliferation during antral follicle growth (2, 3). Interestingly, the transcription factor Sp1 is a target of CDK2 activity (1) and known to play a key role in the regulation of human and rat LHCGR expression (31). Preliminary observations indicate that other Sp1 target genes in granulosa cell-enriched populations from the rat are also regulated in an S-phase-dependent manner, including aromatase (cyp19a) and side-chain cleavage (cyp11a1) (unpublished observations). Furthermore, estrogen cooperates with FSH to increase the expression of LHCGR (15); thus some or all of the observed LHCGR expression during G1-S transit could be due in part to estrogen and/or estrogen receptor. This hypothesis is supported by data suggesting that estrogen can promote the movement of bovine granulosa cells into S phase of the cell cycle (26). Alternatively, release of the transcription factor(s) E2F1-5 through the actions of CDK2 could regulate LHCGR expression. Regardless of the mechanism, the regulation of LHCGR mRNA as a consequence of S phase progression suggests a physiological mechanism to prevent premature luteinization by limiting the expression of LHCGR (and other key genes) during the late stages of antral follicle growth.