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The Mouse Follicle Microenvironment Regulates Antrum Formation and Steroid Production: Alterations in Gene Expression Profiles[1]

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ABSTRACT

Folliculogenesis is a coordinated process, and the genes that regulate development are difficult to investigate in vivo. In vitro culture systems permit the assessment of individual follicles during development, thereby enabling gene expression patterns to be monitored during follicle development. Mouse multilayered secondary follicles (150–180 μm in diameter) were cultured in three-dimensional matrices of varying physical properties for up to 8 days. During this period of follicle growth in vitro, antrum formation and steroid production were monitored, and mRNA was isolated. The expression levels of genes (*Star*, *Cyp11a1*, *Cyp17a1*, *Hsd3b1*, *Cyp19a1*, *Fshr*, *Lhcgr*, *Aqp7*, *Aqp8*, *Aqp9*, and *Hif1a*) were measured and correlated to follicle developmental status. Follicles that developed an antrum and produced appropriate levels of estrogen and progesterone had unchanging expression of *Star*, *Aqp7*, *Aqp8*, and *Hif1a* and a 34-fold increase in *Cyp19a1* expression at Day 8 of culture and had elevated *Lhcgr* at Days 6 and 8 of culture. Follicles that were healthy but did not form an antrum or produce appropriate levels of steroids, however, demonstrated increasing levels of *Star*, *Aqp7*, *Aqp8*, and *Hif1a* and a 15-fold increase in *Cyp19a1* at Day 8 of culture, and *Lhcgr* levels were not elevated until Day 8 of culture. To our knowledge, this study provides the first temporal analysis of gene expression using individual culture in alginate hydrogels that correlates growth and steroidogenesis during follicle development and identifies expression patterns in healthy follicles and in developmentally disadvantaged follicles.

follicle, gene regulation, in vitro follicle development, ovary

INTRODUCTION

Folliculogenesis is a coordinated process driven by signals from ovarian cells and from the anterior pituitary. Following activation from the ovarian reserve, follicle growth is characterized by an increase in oocyte size and by proliferation of the surrounding granulosa cells. This growth is accompanied by differentiation of the granulosa and theca cells, leading to antrum formation and increased steroidogenic function. Folliculogenesis concludes when a mature cumulus-oocyte complex is ovulated from an antral follicle following the luteinizing hormone (LH) surge. Regulation of this process occurs through paracrine and endocrine factors, the follicular extracellular matrix, and communication between the follicular compartments (oocyte, granulosa, and theca cells) [1]. Investigating this complex sequence of events has been difficult in vivo because of the limited ability to track or monitor individual follicles. In vitro follicle culture systems allow for the monitoring of individual follicles and provide a model to investigate this complex sequence of events.

In vitro culture systems aim to mimic the native ovary by providing an environment that supports and directs follicle growth and differentiation [1]. The following two general approaches have been taken to follicle culture [2]: an attached follicle approach in which follicles are allowed to attach and spread on a two-dimensional surface [3–11] and a three-dimensional intact follicle approach in which follicles maintain their native architecture [1, 12–27]. While both approaches have produced live offspring [24, 28, 29], three-dimensional culture systems maintain the follicular architecture, thereby preserving cell-cell interactions and paracrine signaling that direct follicle development [1, 21, 30]. Our laboratory has utilized alginate, a naturally occurring hydrogel derived from brown algae, as a supportive three-dimensional follicle culture matrix. This three-dimensional alginate culture system supports follicle survival and growth [23, 31], while maintaining the transzonal projections that facilitate granulosa cell-oocyte communication within the follicle [15]. The process of in vitro follicle growth and subsequent in vitro maturation of the oocyte has been used with a mouse model to produce high-quality oocytes that can be fertilized and give rise to offspring that are healthy and fertile [24].

Prior work using the alginate culture system showed that the physical properties of the follicle microenvironment alter the developmental fate of the encapsulated follicle, leading to the delineation of specific physical properties of the matrices that are permissive or nonpermissive to follicle development [23, 31]. Follicle survival is unaffected by the environmental stiffness; however, follicle growth, antrum formation, and egg quality are all affected negatively by nonpermissive alginate matrices. The patterns of gene expression associated with follicle differentiation (antrum formation and egg quality), and not merely somatic cell survival and proliferation, have not been well characterized, as these processes occur simultaneously. This culture system provides a powerful opportunity to systematically investigate gene expression in specific cellular processes. In this study, we investigate the dynamic expression profiles of genes that are associated with steroid production and antrum formation, two processes involved in the first step of follicle growth. The genes investigated include

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TABLE 1. Primer details for RT-PCR analysis.

Gene symbol	Gene name	Taqman assay ID
Aqp7	Aquaporin 7	Mm00431839_m1
Aqp8	Aquaporin 8	Mm00431846m1
Aqp9	Aquaporin 9	Mm00508094_m1
Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1	Mm00490735_m1
Cyp17a1	Cytochrome P450, family 17, subfamily a, polypeptide 1	Mm00484040_m1
Cyp19a1	Cytochrome P450, family 19, subfamily a, polypeptide 1	Mm00484049_m1
Fshr	Follicle stimulating hormone receptor	Mm00442819_m1
Hif1a	Hypoxia inducible factor 1, alpha subunit	Mm00468869_m1
Hsd3b1	Hydroxy-delta-5-steroid dehydrogenase	Mm00476184_g1
Lhcgr	Luteinizing hormone/choriogonadotropin receptor	Mm00442931_m1
Star	Steroidogenic acute regulatory protein	Mm00441558_m1

Star, *Cyp11a1*, *Cyp17a1*, *Hsd3b1*, *Cyp19a1*, *Fshr*, *Lhcgr*, *Aqp7*, *Aqp8*, *Aqp9,* and *Hif1a*. Results of these experiments suggest a direct link between the local environment and follicle function and may provide a tool to understand follicle dysfunction.

MATERIALS AND METHODS

Animals and Materials

Follicles were isolated from prepubertal 16-day-old female F_1 hybrids (C57BL/6 × CBA). Breeding pairs and prepubertal animals were housed in a temperature- and light-controlled environment (12L:12D) and were provided with food and water ad libitum. Animals were fed phytoestrogen-free Teklad Global irradiated 2919 chow (Harlan Laboratories, Indianapolis, IN), which does not contain alfalfa or soy meal. Animals were treated in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with protocols approved by the Institutional Animal Care and Use Committee at Northwestern University. Unless otherwise noted, all media formulations were purchased from Invitrogen (Carlsbad, CA) and all chemicals from Sigma-Aldrich (St. Louis, MO).

Alginate Hydrogel Preparation

Sodium alginate (55%–65% guluronic acid) was generously provided by FMC BioPolymers (Philadelphia, PA). To remove impurities, alginate was dissolved in deionized water to a concentration of 1% (w/v) and purified with activated charcoal (0.5 g of charcoal per gram of alginate powder). To sterilize, the alginate solution was filtered through 0.22-μm filters (Millipore, Billerica, MA), lyophilized within Steriflip conical tubes to maintain sterility (Millipore), and sterilely aliquoted. Aliquots were reconstituted 24 h before use with sterile calcium- and magnesium-free 1× PBS to concentrations of 1.5% and 0.5% (w/v).

Alginate Characterization

Alginate shear elastic modulus was measured at 25°C using a Paar Physica MCR rheometer (Anton Paar, Graz, Austria) with parallel-plate geometry and a 50-mm diameter set to a gap of 1 mm. Data were collected using Paar Physica US200 software (Anton Paar). Each alginate solution was quickly blended with a 40% (w/v) $CaSO_4$ slurry (40 μl of $CaSO_4$ slurry per milliliter of alginate) and extruded onto the lower plate of the rheometer. The upper plate was immediately lowered to a distance of 1 mm from the lower plate, and the alginate was allowed to cross-link between the plates for 2 h. Storage moduli were determined by oscillatory shear experiments performed with a strain amplitude of 1% and an oscillation frequency of 10 rad/sec, which is within the linear viscoelasticity region as verified by strain and frequency sweeps following each measurement.

Follicle Isolation, Encapsulation, and Culture

Multilayered secondary follicles (150–180 μm in diameter) were mechanically isolated from the ovaries of 16-day-old C57BL/6 × CBA F_1 female mice as previously described [21]. Single follicles were then transferred into 2–3-μl droplets of alginate at various concentrations (1.5% and 0.5%) on a propylene mesh (0.1-mm opening; McMaster-Carr, Atlanta, GA). The droplets were then immersed in a calcium-containing solution (50 mM $CaCl_2$ and 140 mM NaCl) by immersing the mesh (for 1.5% alginate) or by inverting and tapping the mesh (for 0.5% alginate) as previously described [23]. Alginate beads were allowed to cross-link in the calcium solution for 2 min and were then rinsed for 10 min in growth medium composed of alpha minimum essential medium supplemented with 10 mIU/ml of recombinant human follicle-stimulating hormone (Organon, Roseland, NJ), 3 mg/ml of bovine serum albumin (MP Biomedicals, Irvine, CA), 1 mg/ml of bovine fetuin, 5 μg/ml of insulin, 5 μg/ml of transferrin, and 5 ng/ml of selenium. Alginate beads, each containing a single follicle, were then placed in individual wells of a 96-well plate containing 100 μl of growth medium. Before use, fetuin was dialyzed extensively against embryo culture-grade water and lyophilized. Throughout isolation, encapsulation, and culture, the follicles were maintained at 37°C and at pH 7.

Follicles were cultured for 2–8 days at 37°C with 5% CO_2 in air containing 21% oxygen. Half of the culture media (50 μl) was exchanged every 2 days, and the conditioned media were stored at −80°C for hormonal analysis. Follicle images were collected every 2 days using a Leica DM IL light microscope (Leica, Wetzlar, Germany) equipped with phase objectives, a heated stage, a Spot Insight 2 Megapixel Color Mosaic camera, and Spot software (Spot Diagnostic Instruments, Sterling Heights, MI). Follicle diameters were measured using ImageJ software [32] (National Institutes of Health, Bethesda, MD) as previously described [23]. Growth data represent only those follicles cultured for the full culture period of 8 days. Follicles were classified as dead if the oocyte was no longer surrounded by a layer of granulosa cells, the granulosa cells had become dark and fragmented, and/or the follicle had decreased in size. At the end of culture (Days 2, 4, 6, and 8), the follicle media were replaced with 100 μl of Liebovitz L-15 media containing 10 U/ml of alginate lyase for 30 min at 37°C. Follicles were removed from the degraded alginate and transferred into clean microcentrifuge tubes with a minimal amount of media. The follicles were pooled by culture conditions (21–43 follicles pooled) and were flash frozen in liquid nitrogen and stored at −80°C for subsequent RNA isolation.

Follicle Sectioning and Hematoxylin-Eosin Staining

Following culture, follicles in alginate beads were removed from culture wells and fixed overnight at 4°C in solution containing 4% paraformaldehyde, 0.1 M sodium cacodylate, 0.1 M sucrose, and 10 mM $CaCl_2$ to prevent alginate degradation. Following fixation, follicles were dehydrated through increasing ethanol solutions and embedded in paraffin blocks using an automated tissue processor (Leica, Manheim, Germany). Serial 5-μm sections were cut and stained with hematoxylin-eosin to examine follicle morphology.

Hormone Assays

Progesterone and 17β-estradiol in conditioned media were measured using commercially available radioimmunoassay kits (progesterone; Diagnostic Products Corporation, Los Angeles, CA; and 17β-estradiol; Diagnostic Systems Laboratories, Inc., Webster, TX). The sensitivities for the progesterone and estradiol assays are 0.1 ng/ml and 10 pg/ml, respectively. To obtain sufficient media for each assay, media collected from follicles in identical alginate conditions were pooled for each time point (≥12 samples pooled per measurement). Six independent measurements for each steroid at each time point were performed. Assays were performed at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core, Charlottesville, supported by NIH U54 HD28934.

Characterization of Gene Expression

Following 2, 4, 6, and 8 days of culture, follicles were isolated from alginate beads and were flash frozen in liquid nitrogen as already described.

TABLE 2. Comparison of follicle growth and survival in permissive and nonpermissive alginate matrices.

Alginate density (% w/v)	Follicles (N)	Day 8 survival rate (%)	Diameter increase at Day 8 (%)*
0.5 (permissive)	441	84.3	91.9 ± 6.6†
1.5 (nonpermissive)	433	82.7	60.4 ± 5.4

* Error represents SEM.
† Significant difference.

RNA was purified from follicles using the Absolutely RNA Microprep Kit (Stratagene, Cedar Creek, TX) according to the manufacturer's protocol. RNA quality was verified using the RNA 6000 Nano Chip Assay and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The RNA was reverse transcribed into first-strand cDNA (SuperScript First-Strand Kit; Invitrogen) using random hexamers according to the manufacturer's protocol and was stored at −80°C. Expression of *Star*, *Hsd3b1*, *Cyp17a1*, *Cyp19a1*, *Fshr*, *Lhcgr*, *Hif1a*, *Aqp7*, *Aqp8*, and *Aqp9* was measured. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as an endogenous control. All real-time PCR experiments were performed using the TaqMan gene expression system (Applied Biosystems, Foster City, CA), including TaqMan probes, as summarized in Table 1. Cycles were completed, and results were analyzed using the Applied Biosystems ABI7700 standard program. In this program, samples were warmed to 50°C for 2 min (once), heated to 95°C for 10 min (once), and then cycled 40 times as follows: 95°C for 15 sec and 60°C for 60 sec.

Statistical Analysis

Statistical calculations were performed using JMP 4.0.4 software (SAS Institute, Cary, NC). Statistical significance for follicle size measurements, steroid levels, and PCR results was analyzed using two-way ANOVA with repeated measures or one-way ANOVA followed by Tukey honestly significant difference for single time points. Chi square analysis was used to analyze categorical data.

RESULTS

Permissive and Nonpermissive Follicle Culture

Initial studies were performed to define permissive and nonpermissive culture conditions. Follicles were cultured in 0.5% and 1.5% alginate for up to 8 days (Table 2), and survival and growth were monitored during this time. The mechanical properties of alginate preparations were characterized and indicated that the 1.5% alginate hydrogel had a mean ± SEM shear modulus of 1300 ± 130 Pa, which is an order of magnitude greater than the shear modulus of the 0.5% alginate hydrogel (121 ± 7 Pa) (Fig. 1A). The mechanical properties and solids content of alginate hydrogels have previously been characterized as influencing follicle development in vitro [31]. Follicle survival rates were 84% in 0.5% alginate matrices and 83% in 1.5% alginate matrices, demonstrating that follicle survival was not significantly affected by the alginate condition (Table 2). Follicles also increased in size in both alginate conditions (Fig. 1B). Follicles in 0.5% alginate reached a mean ± SEM diameter of 381 ± 3 μm, which is significantly greater ($P < 0.05$) than follicles cultured in 1.5% alginate, which reached a final diameter of 295 ± 5 μm. Based on these results, we refer to the 0.5% alginate matrix as permissive and the 1.5% alginate matrix as nonpermissive.

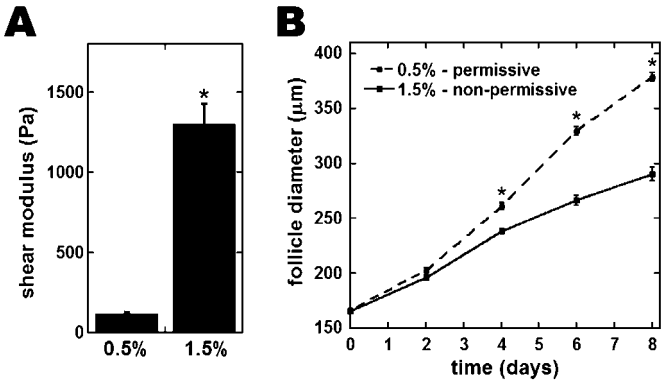

FIG. 1. Alginate stiffness and follicle growth. **A**) Shear modulus measurements of 0.5% and 1.5% alginate hydrogels. **B**) Growth of multilayered secondary follicles cultured in permissive and nonpermissive matrices for 8 days. Error bars represent SEM. *Significance relative to 1.5% alginate ($P < 0.05$).

Steroid Levels

Estradiol levels were higher in follicles cultured in permissive matrices, reaching a level of 3.7 ng/ml by Day 8, while those cultured in nonpermissive matrices produced estradiol at a level of 1.1 ng/ml by Day 8 of culture (Fig. 2A). Progesterone levels increased throughout culture (Fig. 2B) but were not significantly different until Day 8, at which point levels in follicles cultured in nonpermissive matrices were higher. The morphology of the granulosa cells within follicles in nonpermissive matrices was subsequently investigated (Fig. 2C). In the cross section, columnar granulosa cells face the squamous cells in the periphery. The follicle morphology is consistent with in vivo observations, and the nuclei area:somatic cell area ratio is similar. The cobblestone appearance of the granulosa cells in the inset of the figure is also consistent with in vivo observations. Taken together, these observations indicate that the follicle is healthy, which is further supported by previous results in which follicles grown under these conditions supported healthy egg development and live, healthy offspring [22, 24].

Gene Expression in Steroidogenesis

Steroid hormones were detectable in the media at Day 4 of culture and increased throughout culture. The levels detected varied with time and with the matrix condition; thus, we examined expression of the following genes related to steroidogenesis during the time course of follicle culture: *Star*, *Cyp11a1*, *Hsd3b1*, *Cyp17a1*, *Cyp19a1*, *Fshr*, and *Lhcgr*. *Star* was significantly upregulated in mature follicles (Fig. 3A) at Days 6 and 8 of culture in 1.5% alginate but not in 0.5% alginate cultures. *Cyp11a1* expression decreased throughout culture for the permissive matrix and was decreased only at Days 4 and 8 for the 1.5% alginate condition (Fig. 3B). *Hsd3b1* expression, required for progesterone and androstenedione production, was upregulated at Day 8 for the nonpermissive matrix (Fig. 3C). *Cyp17a1* expression was highest at Day 2 of culture (Fig. 3D); however, the mean expression levels in 0.5% alginate were decreased at all time points, although only Day 8 was statistically significant. Expression of *Cyp17a1* in 1.5% alginate was highest at Day 2, lowest at Day 4, and increased after Day 4. *Cyp19a1* expression demonstrated a large increase in the 0.5% and 1.5% alginate conditions at Day 8 (Fig. 3E). Expression was significantly upregulated in follicles cultured in 0.5% alginate at Days 6 and 8 (34-fold at Day 8), while upregulation in 1.5% alginate did not occur at Day 6 and was upregulated to a lesser extent (15-fold) than the permissive matrix at Day 8 ($P < 0.05$). *Fshr* expression was not significantly affected by alginate condition and did not change during culture (Fig. 3F). *Lhcgr* expression, however, increased as the follicles matured and demonstrated a significant increase in expression in follicles cultured in 0.5% alginate at Days 6

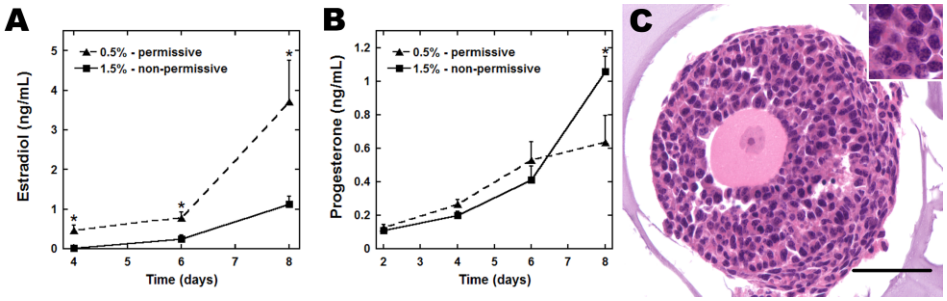

FIG. 2. Steroid production and morphology of multilayered secondary follicles for 8 days in culture. Estradiol production (**A**), progesterone production (**B**), and follicle cross section (**C**) from Day 8 of nonpermissive culture. Bar = 100 μm; inset is 2× original magnification. Estradiol levels were below the detectable limits of the assay at Day 2 of culture. Error bars represent SEM. *Significance relative to 1.5% alginate ($P < 0.05$).

and 8 in 0.5% alginate and at Day 8 in 1.5% alginate (Fig. 3G) ($P < 0.05$).

Antral Cavity Formation

No antra were observed at Days 0, 2, and 4 of culture (Fig. 4A). At Days 6 and 8, the number of follicles with antra was significantly greater in the 0.5% alginate than in the 1.5% alginate condition, with 86% of follicles cultured in 0.5% alginate and only 6% of follicles cultured in 1.5% alginate forming an antrum by Day 8. Phase images and histological sections demonstrate the presence of an antrum in follicles cultured in permissive matrices but not in nonpermissive matrices (Fig. 4, B–E).

Antrum-Regulating Gene Expression

To investigate the role of transcription factors involved in cellular response to lack of oxygen during follicle development and antrum formation, expression of *Hif1a* was assessed. *Hif1a* expression remains unchanged in the 0.5% alginate condition but is significantly increased in the 1.5% alginate condition at Days 6 and 8 of culture (Fig. 5A).

Based on previous findings about the role of aquaporin expression on antrum formation [33], aquaporin 7, 8, and 9 expression was examined by RT-PCR in follicles cultured in 0.5% and 1.5% alginate. Aquaporin 9 was not detected until very high cycle numbers if at all; therefore, reliable expression levels could not be determined. In 1.5% alginate, aquaporin 7 was significantly increased at Day 6 (Fig. 5B), whereas in 0.5% alginate expression was decreased at Days 6 and 8. Aquaporin 8 demonstrated significantly increased expression at Days 6 and 8 for 1.5% alginate (Fig. 5C) and was significantly increased only at Day 8 for 0.5% alginate, although the increase was substantially less compared with 1.5% alginate.

DISCUSSION

During follicle development, the multiple cellular compartments interact via secreted factors and gap junctions to coordinate follicle development. Antrum formation and steroidogenesis are two aspects of this developmental process

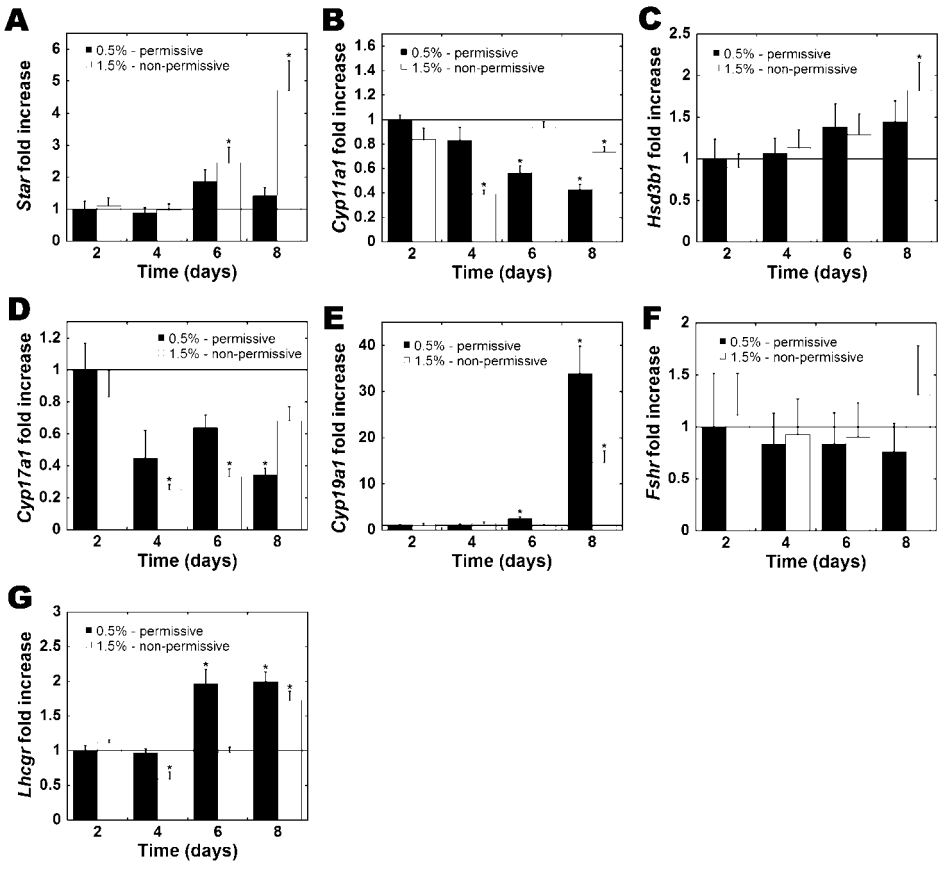

FIG. 3. Steroidogenic pathway gene expression quantified by RT-PCR. **A**) *Star*. **B**) *Cyp11a1*. **C**) *Hsd3b1*. **D**) *Cyp17a1*. **E**) *Cyp19a1*. **F**) *Fshr*. **G**) *Lhcgr*. Results are normalized to expression in permissive matrices at Day 2 of culture. Horizontal lines indicate the normalized expression for the 0.5% condition, from which the fold increase is defined. Error bars represent SD. *Significance relative to Day 2 ($P < 0.05$).

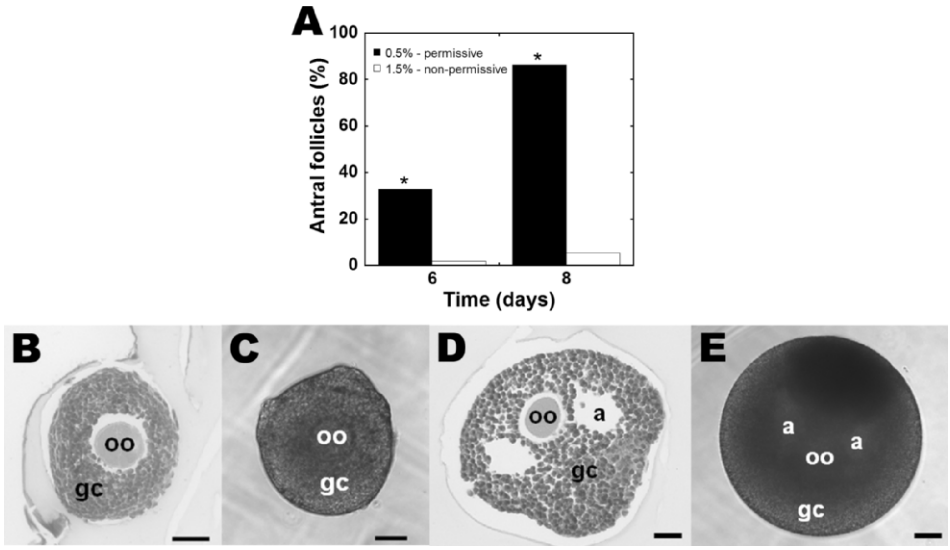

FIG. 4. Antrum formation. **A**) Antrum formation in permissive and nonpermissive matrices at Days 6 and 8 of culture. No antra were observed before Day 6. **B** and **C**) Follicles cultured in nonpermissive matrices. **D** and **E**) Follicles cultured in permissive matrices. Hematoxylin-eosin-stained paraffin sections (**B** and **D**) and phase-contrast images (**C** and **E**). Bar = 50 μm. *Significance relative to 1.5% alginate ($P < 0.05$). oo, oocyte; gc, granulosa cell; a, antrum.

and are influenced by the matrix [31]. Previous studies [23, 31] have demonstrated that altering the matrix physical properties can change the microenvironment from permissive to nonpermissive for follicle growth. Although factors such as hydrogel solids content, mesh size, and degradation influence follicle growth [31], the mechanical properties of the matrix have emerged as a significant regulator of follicle development. Matrices with a shear modulus of less than 250 Pa are regarded as permissive, as a large percentage of follicles cultured in these matrices survive and increase in diameter, develop an antrum, have a steroidogenic profile similar to that of follicles in vivo, and produce metaphase II stage oocytes. Conversely, matrices with a shear modulus greater than 500 Pa are regarded as nonpermissive, as they result in decreased growth and antrum formation rates, as well as altered steroid production, thereby decreasing the oocyte quality [23, 31].

The expression of genes associated with steroidogenesis and antrum formation was subsequently investigated to elucidate their patterns as a function of the matrix properties. In permissive matrices, *Star*, *Aqp7*, *Aqp8*, and *Hif1a* were not upregulated more than 2-fold between Days 0 and 8, there was a 34-fold increase in *Cyp19a1* expression at Day 8 of culture, and there was elevated *Lhcgr* at Days 6 and 8 of culture. This pattern of gene expression is occurring in steroidogenically healthy antral follicles, which produce quality oocytes [23, 31]. Follicles cultured in nonpermissive matrices were healthy but produced less estradiol and more progesterone than follicles cultured in permissive matrices, and only 5.5% of follicles formed antra. These follicles demonstrated increasing levels of *Star*, *Aqp7*, *Aqp8*, and *Hif1a* and only a 15-fold increase in *Cyp19a1* at Day 8 of culture, and *Lhcgr* was not induced until Day 8 of culture. This pattern in gene expression is associated with poor antrum formation, resulting in decreased follicle differentiation and oocyte quality.

The permissive culture environment aims to recreate the environment of the natural ovarian cortex, and it does so in promoting differentiation of granulosa cells, leading to antrum formation and steroidogenesis. Within the ovary, antrum formation correlates with increasing estradiol production and with only moderately increasing progesterone production as the follicle reaches maturity. Permissive in vitro culture supported antrum formation in more than 85% of follicles. These follicles also produce sharply increasing levels of estradiol, with only slight increases in progesterone near the end of the culture period. Steroid synthesis in the ovary is regulated by the activity of the steroidogenic enzymes and by the availability of cholesterol substrate from which to produce steroids. CYP19A1 is predominantly expressed in granulosa cells and is responsible for the conversion of androstenedione to estradiol [34]. During development in vivo, follicles demonstrate increasing *Cyp19a1* expression as they mature and approach ovulation [34, 35]. *Cyp19a1* expression increased more than 30-fold by Day 8 of culture, suggesting that the increase in estradiol production is regulated by enzyme expression at the mRNA level. STAR is critical in regulating the transport of cholesterol required for steroid hormone synthesis [36] and is localized to the theca and interstitial cells until after the LH surge in vivo [37, 38]. In permissive conditions, *Star* levels remained unchanged throughout culture.

In addition to steroidogenic enzymes, gonadotropin receptors have an important role in regulating follicular development and steroidogenesis. In vivo, *Lhcgr* is expressed in mature follicles as they approach ovulation. Upregulation of LHCGR allows the follicle to respond to the LH surge, resulting in ovulation, cumulus mucification, and granulosa cell luteiniza-

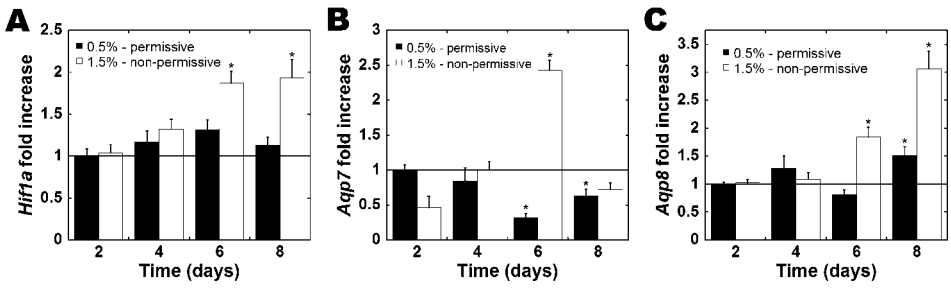

FIG. 5. Expression levels for antrum-related genes. The genes investigated include *Hif1a* (**A**), *Aqp7* (**B**), and *Aqp8* (**C**) expression. Results are normalized to expression in permissive matrices at Day 2 of culture. Error bars represent SD. Horizontal lines indicate the normalized expression for the 0.5% condition, from which the fold increase is defined. *Significance relative to Day 2 ($P < 0.05$).

tion [39]. Therefore, expression of *Lhcgr* during in vitro culture is a sign of follicle maturity. In permissive follicle culture, upregulation of *Lhcgr* occurred at Day 6 of culture, which coordinates with the timing of follicle differentiation and antrum development. Unlike *Lhcgr*, *Fshr* is expressed in both immature and mature granulosa cells [40], and expression levels remain constant throughout follicle development at the mRNA level and at the protein level [41, 42]. Consistent with these in vivo observations, *Fshr* expression was unchanged throughout the duration of culture.

The physically nonpermissive culture matrix disrupts antrum formation and steroidogenic activity. In nonpermissive matrices, antra formed in only 5.5% of follicles. Unlike the permissive cultures, steroidogenesis in nonpermissive matrices was characterized by only slight increases in estradiol production, with sharply increasing levels of progesterone. This pattern of steroidogenesis is regarded as a negative indicator of follicle health [43]. Progesterone levels were similar at Day 6 in permissive and nonpermissive culture conditions, which expressed significantly different levels of *Lhcgr*. Mechanical stresses are known to regulate cell behavior in many cell types [44, 45], and a possible explanation for the observed results is that mechanotransduction signaling events may be regulating progesterone production via alternative pathways. The sharp increase in progesterone production by Day 8 of culture indicates follicular demise or terminal differentiation, potentially due to mechanotransduction in the stiff matrix or due to hypoxic conditions in the center of the follicle. *Cyp19a1* levels were much lower than in permissive culture, demonstrating that the disruption to estradiol production occurs at the mRNA level. Unlike permissive cultures, *Star* expression increased during Days 6 and 8 of culture. The mechanical environment may regulate steroid production, although it does not seem to extend to premature luteinization because we do not observe an increased cytoplasmic:nuclear volume. In addition, similar patterns of endocrine disruption have been observed in other pathological conditions, including cases of exposure of granulosa cells to bisphenol A, which causes increased *Star* expression and progesterone production concurrent with decreased *Cyp19a1* expression and estradiol production [46]. Similar patterns of endocrine disruption indicate that this may be the physiological response of follicles to pathologic environments. Gonadotropin receptor gene expression was also examined, and *Lhcgr* expression did not increase until Day 8 of culture, demonstrating that follicle maturation is delayed in nonpermissive conditions relative to permissive cultures. Similar to permissive cultures, *Fshr* mRNA abundance was unchanged throughout culture, suggesting that change in follicle-stimulating hormone response does not underlie the change in steroidogenesis.

In addition to steroidogenesis, the nonpermissive culture matrix restricted antral cavity formation. Unlike steroidogenesis, however, the genes regulating antrum formation have not been clearly established, and the regulation of antrum formation and its role in folliculogenesis are not well understood. One proposed function of the antrum is to alleviate hypoxia in the center of the growing follicle [47, 48]. As granulosa cells proliferate and forming multiple layers surrounding the oocyte, the avascular interior of the follicle is thought to become increasingly hypoxic [47]. Mathematical modeling has been used to support a model whereby oxygen transport is facilitated by the presence of an antrum [48, 49]. To date, however, no experimental evidence has been produced to support this idea.

As an indicator of hypoxic conditions, expression of *Hif1a* mRNA in developing follicles in permissive and nonpermissive matrices was measured. HIF1A is the regulated component of the HIF1 heterodimer complex, which is a well-known mediator of oxygen homeostasis [50]. *Hif1a* mRNA expression is upregulated in hypoxic conditions in many organs and cells [51, 52], and findings in the ovary show that *Hif1a* mRNA is differentially expressed within ovarian cells and that expression varies at different stages of the cycle [53]. Increasing expression of *Hif1a* in follicles cultured in the nonpermissive three-dimensional culture system suggests that a follicle unable to form an antrum becomes increasingly hypoxic as it continues to grow. Follicles cultured in permissive culture conditions, however, were able to develop an antral cavity, which correlated with unchanging levels of *Hif1a* expression (Fig. 5A). Antrum formation begins between Days 4 and 6 of culture, when follicles reach diameters between 239 and 333 μm. Based on the mathematical model, these follicles are in the predicted size range at which the center of the follicle becomes hypoxic [49], lending support to the mathematical model and to the hypothesis that the antrum functions to alleviate hypoxia in growing follicles.

While alleviation of hypoxia may be the physiological motivation for development of an antrum, the process by which the antrum forms is also not well understood. Recent studies [33, 54] have indicated that aquaporins 7, 8, and 9 regulate water permeability during antrum formation. We hypothesized that antrum formation may be regulated by expression of aquaporins at the mRNA level and examined expression of *Aqp7*, *Aqp8*, and *Aqp9* during permissive and nonpermissive three-dimensional follicle culture. Low levels of *Aqp9* were detected in the system and have previously been reported [55]; therefore, it is likely that regulation of *Aqp9* expression in the ovary cannot be examined at the mRNA level. In permissive conditions that support a high rate of antrum formation, *Aqp7* expression decreased at Days 6 and 8 of culture. *Aqp8* levels in permissive cultures increased significantly at Day 8, but this increase was small relative to the increase in expression in nonpermissive cultures. In nonpermissive culture conditions, *Aqp7* expression was upregulated at Day 6 of culture, and *Aqp8* expression was elevated at both Days 6 and 8 of culture, demonstrating a 3-fold increase in expression by Day 8. Thus, expression of *Aqp7* and antrum formation are negatively correlated during follicle growth. While this result was unanticipated, the negative correlation is not necessarily inconsistent with previous investigations that examined aquaporin protein expression in granulosa cells from equine chorionic gonadotropin-stimulated ovaries at a single time point [33]. Rather, these data may indicate that an induction of gene expression may not occur before antrum formation, but instead must be expressed at a constant level in developing follicles, and that the nonpermissive environment mechanically disrupts the ability of water to enter the follicle. Alternatively, the mechanisms of antrum formation may be altered in the in vitro environment, in which mechanotransduction events may directly affect gene expression, or the process of antrum formation in the earlier study [33] may differ from physiological antrum formation because of exogenous hormonal stimulation. Further investigation of this idea is necessary, and the ability to regulate antrum formation of individual follicles in vitro in the three-dimensional culture system will provide a unique platform with which to investigate the process.

In summary, the results in this study demonstrate that permissive matrices support the coordination necessary for folliculogenesis, while this coordination is disrupted in physically nonpermissive matrices at the mRNA level. The increased quality of follicles cultured in permissive matrices

may indicate that the permissive culture better mimics the natural environment of the ovary. Disruption of steroidogenesis in nonpermissive matrices resulted in decreased levels of estradiol production and in increased levels of progesterone, which are generally viewed to be negative signs of follicle health. This disruption to steroidogenesis was accompanied by decreased *Cyp19a1* expression, delayed *Lhcgr* expression, and upregulation of *Star* in granulosa cells. In addition, follicles cultured in nonpermissive matrices were not able to form an antral cavity, which correlated with increasing levels of *Hif1a* expression. This increased expression was not observed in follicles able to form an antrum in permissive matrices, suggesting that formation of an antrum may alleviate the hypoxic conditions that could lead to *Hif1a* upregulation. Upregulation of aquaporins occurred in follicles unable to form antra and correlated negatively with antrum formation. Taken together, the results of this study provide improved molecular understanding of antrum formation and steroidogenesis in the developing follicle and can potentially assist in identifying mechanisms of follicle dysfunction.

In addition to providing a tool for investigating the regulation of follicle development, in vitro follicle growth and subsequent oocyte maturation may also provide a means to preserve reproductive potential for women with cancer by supporting the maturation of cryopreserved immature follicles to produce mature, fertilizable oocytes. As this system is translated to the human, studies correlating production of secreted factors (such as steroid hormones) to the quality of the follicle and developmental state of the oocyte will be invaluable to determine when the follicle and oocyte are competent to undergo in vitro maturation. Determining proper endocrine patterns and identifying the in vitro culture conditions that support this expression should allow for development of a successful in vitro culture system to support human follicle development.

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