# Title page

Title:

Gonadotropin and Oxygen Regulation of Leukemia Inhibitory Factor Secretion from Rhesus Macaque Granulosa Cells

Title: State the key point of the manuscript. Indicate the species studied and avoid uncommon abbreviations. Maximum 120 characters including spaces.

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Abbreviations:

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Regulation of granulosa cell LIF synthesis

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# **Abstract (250 words)**

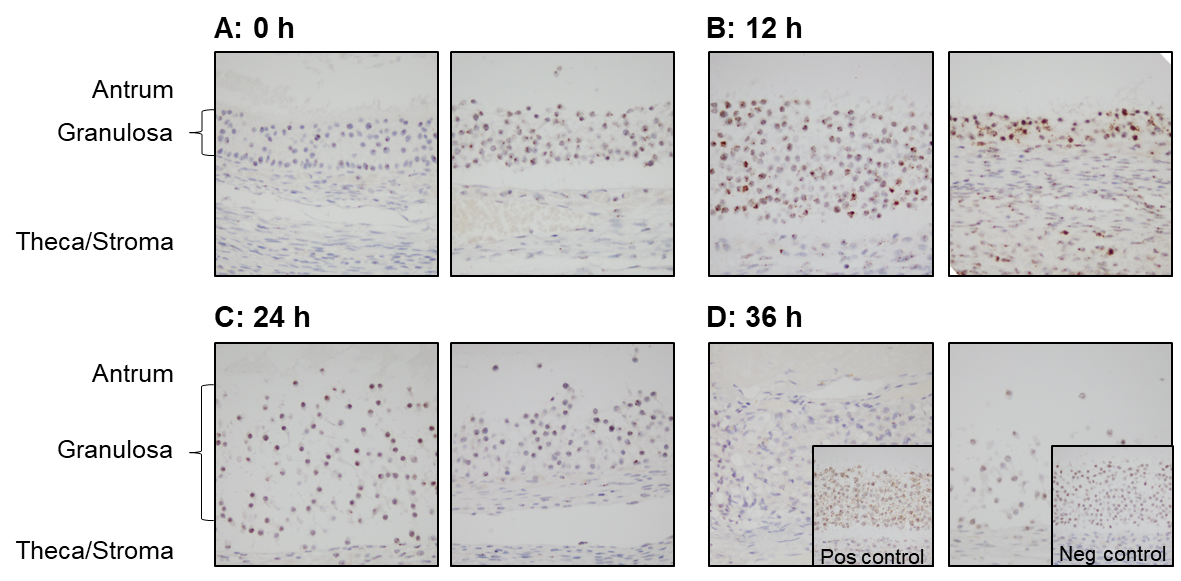
Leukemia inhibitory factor (LIF) is required for rhesus macaque ovulation. However, it is unclear which cells within the ovarian follicle produce LIF and what factors control its synthesis. Rhesus ovaries collected prior to (0 h) or 12, 24, and 36 h after receiving an ovulatory bolus of human chorionic gonadotropin (hCG) were assessed by RNAScope to determine LIF mRNA cellular localization. Media was assessed for LIF, vascular endothelial growth factor (VEGF), and progesterone (P4) after treating rhesus macaque non-luteinized and luteinized granulosa cells and KGN cells, a steroidogenic human granulosa-like tumor cell. Culture treatments included hCG (0, 40 IU/mL) alone or with varying concentrations of follicle stimulating hormone (FSH; 8.2, or 40 mIU/mL). The different treatments were incubated in the presence of low and high oxygen concentrations (1%, or 20%, respectively). LIF (0, or 1 ng/mL for luteinized granulosa cells), and forskolin (0, 1 µM for KGN cells). Granulosa cells of the dominate follicle express peak LIF mRNA levels 24h post ovulatory stimulus, visualized by RNAscope. Rhesus non-luteinized granulosa cells increase LIF secretion in response to hCG (3.4-fold) and FSH (XX-fold). However, luteinized granulosa cells did not secrete LIF either basally or in response to hCG or FSH. Non-luteinized and luteinized granulosa cells both secrete VEGF in response to hCG and FSH. Rhesus non-luteinized granulosa cells increase secretion of LIF when cultured in 1% O2, furthermore LIF secretion increased synergistically (XX-fold) when treated with both hCG and 1% O2.

Rhesus non-luteinized granulosa cells respond to in vitro hCG treatment by: 1) increasing LIF secretion 3.4-fold relative to cells cultured in the absence of hCG, 1%, 2) When non-luteinized granulosa cells were cultured in 1% O2, both basal and hCG-stimulated LIF secretion were increased 1.3-fold compared to 20% O2. The combination of hCG and 1% O2 interacted synergistically to increase LIF secretion. Incubating NLGCs in the presence of both FSH and hCG did not increase LIF or progesterone levels above what was observed when the cells were treated with hCG alone. Thus, in vivo it is likely the granulosa cells are a significant source of the LIF that is found in follicular fluid after an ovulatory stimulus.

# Graphical Abstract

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# Figures and Figure Legends



### Figure 1 – Determination of Ovarian Cell Type Responsible for LIF Secretion

RNAscope analysis of rhesus macaque follicles collected at indicated times after ovulation induction by hCG, the ovarian sections were probed for LIF mRNA expression. Panel A: Ovaries collected prior to hCG administration (0 h), Panel B: ovaries collected 12 h after hCG treatment, Panel C: ovaries collected 24 h after hCG. Panel D: ovaries collected 24 h after hCG, insets show positive control probe (PPIB, cyclophilin B), and negative control probe (DapB, dihydrodipicolinate reductase, only found in bacteria).



### Figure 2 – Gonadotropin regulation of rhesus macaque granulosa cells

Nonluteinized granulosa cells (left panels) and 36 h luteinized granulosa cells (right panels) cultured for 24 h at 20% O2 with 0 IU/mL hCG or 40 IU/mL and 0, 0.5, or 2.5 ng/mL FSH. Panel A: LIF concentrations in culture media (pg/mL). Panel B: VEGF concentrations (pg/mL). Panel C: Progesterone concentrations. Panel D: Normalized mRNA expression of hCG treatment marker HSD3B2, note the different scales for non-luteinized and luteinized granulosa cells. Scatterplots of values are superimposed on bar graphs indicating condition mean, 0 IU/mL hCG (●), 40 IU/mL hCG (○), n = 4. Significance was determined using three-way ANOVA with matching, α = 0.05

P-values determined for main effects and interactions of nonluteinized granulosa cells: LIF (FSH = 0.006, hCG = 0.03, FSH x hCG = 0.11), VEGF (FSH = 0.002, hCG = 0.004, FSH x hCG = 0.005), progesterone (FSH = 0.007, hCG = 0.02, FSH x hCG = 0.01), and HSD3B2 mRNA (FSH = ?, hCG = ?, FSH x hCG = ?). P-values determined for main effects and interactions of luteinized granulosa cells: LIF (FSH = 0.9, hCG = 0.3, FSH x hCG = .2), VEGF (FSH = <0.0001, hCG = 0.02, FSH x hCG = 0.007), progesterone (FSH = 0.1, hCG = <0.0001, FSH x hCG = 0.02), and HSD3B2 mRNA (FSH = ?, hCG = ?, FSH x hCG = ?).

Conditions that differ significantly are labeled with no letters in common, significance determined by Tukey’s multiple comparisons test, α = 0.05.

### Figure 3 – Oxygen regulation of rhesus macaque granulosa cells



Nonluteinized granulosa cells (NLGC, left panels) and 36 h luteinized granulosa cells (LGC, right panels) cultured for 24 h at 20% O2 or 1% O2 with 0 or 40 hCG (IU/mL). Panel A: Culture media concentration of LIF (pg/mL). Panel B: Culture media concentration of VEGF (pg/mL). Panel C: Progesterone concentration in culture media. Panel D: Normalized mRNA expression of hypoxia marker KDM3A. n = 4

### Figure 4 – Gonadotropin and oxygen regulation of KGN human granulosa tumor cell line



KGN cells cultured for 24 h at 20% O2 or 1% O2 with 0 or 40 hCG (IU/mL). Panel A: Culture media concentration of LIF (pg/mL). Panel B: Progesterone concentration in culture media. Panel D: Normalized mRNA expression of hypoxia marker KDM3A and hCG treatment marker HSD3B2.

### Figure 5 – LIF regulation of rhesus macaque granulosa cells



Nonluteinized granulosa cells (NLGC, left panels) and 36 h luteinized granulosa cells (LGC, right panels) cultured for 24 h with 0 or 1 ng/mL LIF at 20% O2 or 1% O2 with 0 or 40 hCG (IU/mL). Panel A: VEGF concentration (pg/mL) in culture media. Panel B: Progesterone concentration in culture media. Panel C: Normalized mRNA expression of LIF treatment marker SOCS3. Panel D: Normalized mRNA expression of hypoxia marker KDM3A and hCG treatment marker HSD3B. n = 4

# Introduction

Luteinizing hormone (LH) stimulates ovulation and induces an inflammatory-type reaction in the follicle, which is necessary for fertility. During ovulation, LH induces secretion of critical intrafollicular factors, which notably include inflammatory cytokines. However, the functional roles for most ovulation-associated cytokines are unknown in non-human primates and humans. There is a critical need to determine the function of LH-stimulated follicular cytokines in order to determine essential pathways for ovulation that could serve as targets for regulation of female fertility.

Follicular LIF levels increase 15-fold in rhesus macaques [1] and humans [2] after an ovulatory stimulus. We determined that the complete canonical LIF-signaling pathway is present in the rhesus macaque follicle during ovulation [3]. Members include the LIF receptor, co-receptor (gp130), and the LIF-activated transcription factor STAT3 (signal transducer and activator of transcription 3). Most notably, LIF signaling is required for rhesus macaque ovulation, which our laboratory recently published [1]. Although LIF follicular concentrations are positively correlated with embryo quality, the downstream functional LIF effects are unknown in the follicle.

Thus, the objective of this study was to determine factors that promote LIF signaling in rhesus macaque granulosa cells. The hypothesis was that secretion of the cytokine leukemia inhibitory factor (LIF) was regulated by processes known to be important for ovulation, specifically LHCGR activation and low oxygen tension. Additionally, LIF activates canonical inflammatory transcription factors and modulates progesterone biosynthesis in the rhesus macaque follicular granulosa cells.

# Materials and Methods

## Animal and Culture Protocols

All animal protocols have been previously described in detail including the housing and general care of rhesus macaques(*Macaca mulatta*) [4], monitoring of serum hormone levels [1], controlled ovulation [5], controlled ovarian stimulation [6], rhesus granulosa cell isolation [7], and rhesus granulosa cell culture [7,8]. Definitions and key parameters are briefly described below.

Controlled ovulation allows for the natural selection and gonadotropin-supported development of a single follicle and allows for precision treatment of ovulatory stimuli and subsequent collection of the follicle. Adult female monkeys menstrual cycles were monitored by menses and serum P4 and E2. When E2 levels reached 95-120 pg/mL a GnRH antagonist is administered to prevent a spontaneous LH surge, and FSH and hLH (30 IU each; Repronex, Ferring Pharmaceuticals, Parsippany, NJ) were administered over the next 36 h to support follicle development. On the third day of the protocol, the animals received an ovulatory bolus of hCG (1000 IU hCG; Novarel; Ferring Pharmaceuticals). The ovary containing the dominate follicle was collected at 12 h, 24 h, or 36 h after hCG administration; as well, ovaries were collected from animals that did not receive hCG (0 h). Ovaries were fixed by paraformaldehyde/sucrose, paraffin embedded, sectioned, and imaged as previously described [1].

Controlled ovarian stimulation is a technique used to induce ovulation by multiple ovarian follicles for collection of multiple oocytes, and large quantities of granulosa cells. Within 3 days of menstruation onset, animals received twice daily injections of hFSH (20 IU, 0800 and 1600 h) for six days. **For non-luteinized granulosa cells**, on the seventh day FSH and hLH were administered (50 IU each, 0800 and 1600 h; Menopur, Ferring Pharmaceuticals, Parsippany, NJ) along with the GnRH antagonist acyline (75 µg/kg, 1600 h). On the eight day, cumulus oocyte complexes and granulosa cells were aspirated. **For luteinized granulosa cells**, on the seventh day animal received FSH and hLH (50 IU each, 0800 and 1600 h; Menopur, Ferring Pharmaceuticals, Parsippany, NJ) along with the GnRH antagonist acyline (75 µg/kg, 1600 h). On the eighth day, the animals received FSH and hLH (50 IU each, 0800 and 1600 h; Menopur, Ferring Pharmaceuticals, Parsippany, NJ) and an ovulatory bolus of hCG (1100 IU hCG; Novarel; Ferring Pharmaceuticals, Parsippany, NJ). Thirty-six hours after hCG administration, cumulus oocyte complexes and granulosa cells were aspirated by laproscopic techniques 36 h after hCG administration for luteinized granulosa cells.

Granulosa cells were isolated from follicular aspirates by first removing oocytes, the remaining cells were pelleted and resuspended in Ham F10 medium. Granulosa cells were cleared of red blood cells by layering the cell suspension over 30% Percol in Hanks Balanced Salt Solution and centrifuging for 30 min at 500 xg. Cells at the interface were collected, diluted 1:5 in Ham F-10 and pelleted by centrifugation at 170 xg.

Rhesus granulosa cells were assessed for viability and cultured in fibronectin-coated 48-well plates (5E04 live cells/well; XXX live cells/mL, XXX live cells/cm2) in DMEM/F12 [27.6 µg/mL human low density lipoproteins (Athens Research and Technology, 12-16-120412, Athens, GA), ITS supplement (5 µg insulin, 5 µg transferrin, 5 ng selenium, Corning, 354351), 2 µg/ml aprotinin (Sigma, A3428), 1.5 g/L sodium bicarbonate, 100 U penicillin/100 lg/ml streptomycin, 0.1% BSA, phenol red, pH  7.4] in a humidified incubator at 37 °C/5% CO2. Treatments were added within 2 h of plating. Twenty-four hours after treatments were added, culture media was collected and TRIzol was added directly to wells (100 µL/well). Media was immediately frozen at -20 until analysis. Plates were immediately frozen at -80 °C until analysis.

IUCAC Approvals:

## RNAScope

Slides containing PFPE ovarian sections were assessed for LIF mRNA presence and localization using RNAxcope, a technology for detection amplified in situ hybridization. All in situ hybridization materials were obtained from Advanced Cell Diagnostics (Hayward, CA). The target probes used were: LIF, cyclophilin B (positive control) and bacterial dihydrodipicolinate reductase (negative control). Staining parameters included boiling time of XXX m, protease digestion of XXX concentration for XXX m, and probe incubation time of XXX m. The slides were mounted using DPX mountant (Sigma-Aldrich, St. Louis, MO), after staining and dehydrating. Slides were imaged using a 40x objection on an Olympus BX40 scope with an Olympus DP72 camera.

## Culture Media Analysis

The Endocrine Technologies Support Core Laboratory (ETSL) at the ONPRC performed the culture media ELISAs for LIF (item and vendor), VEGF (item and vendor), and progesterone (item and vendor) according to manufacturers’ instructions and with appropriate internal controls and validation methods.

## qPCR

RNA was prepared from TRIzol extracts from cultured cells using Zymo RNA Extraction Columns (Item #, Zymo, Vendor Location) with an on-column DNA digestion of 45min at 37 °C. RNA integrity was assessed by Bioanalyzer and Qubit RNA Quality kit. RNA samples (200 ng/sample) were reverse transcribed using SuperScript IV (Invitrogen) to create cDNA. Samples were analyzed for target gene expression of HSD3B2, KDM3B, or SOCS3 (FAM reporter) simultaneous with an internal reference gene mitochondrial ribosomal protein S10 (MRPS10, VIC reporter) using XXX master mix on ABI 7200 Fast qPCR machine, probe and primer information can be found in **Table 1**. Target gene expression was normalized to the MRPS10 levels and relative gene expression to control (untreated) samples was determined within samples from a single animal.

## Statistics

Data was compiled, graphed, and statistically tested using “R”. R code and complete data are available in the supplemental information.

# Results

## Localization of LIF mRNA in rhesus macaque ovaries

Localization of LIF mRNA in rhesus macaque ovaries during a time series post-ovulatory stimulation was performed using RNAScope. Prior to an ovulatory stimulus (0 h), LIF mRNA levels were low (Figure 1A). Twelve hours after stimulation of ovulation, LIF had the most intense staining (of the time series investigated), predominately localized within the granulosa cells (Figure 1B). LIF presence in rhesus ovaries continued to be detectable 24 h after an ovulatory stimulus, but had less intense staining than 12 h, though still predominately localized with granulosa cells (Figure 1C). By 36 h post-stimulation, LIF staining intensity had returned to pre-stimulation levels (Figure 1D).

## Gonadotropin regulation of LIF secretion by rhesus granulosa cells

Rhesus non-luteinized granulosa cells secreted LIF in response to hCG, and FSH, additinally hCG and FSH synergistically increased LIF secretion (Figure 2A, left panel). In contrast, rhesus luteinized granulosa cells secreted little to no LIF basally, and neither hCG or FSH stimulated LIF secretion. However, both non-luteinized and luteinized granulosa cells secreted VEGF (Figure 2B) and progesterone (Figure 2C) as expected. Granulosa cell expression of HSD3B2 was used to verify appropriate gonadotropin signaling. Interestingly, non-luteinized granulosa cells showed a high degree of variability in HSD3B2 gene expression in response to hCG and FSH (range: 10-fold to 50-fold over control), whereas luteinized granulosa cells had small but low variability in HSD3B2 gene expression changes (2.5-fold over control) in response to hCG and FSH.

## Oxygen regulation of LIF secretion by rhesus granulosa cells

Non-luteinized granulosa cells secreted more LIF in the presence of 1% O2 compared to 20% O2, and low oxygen and hCG synergistically increased LIF secretion (Figure 3A, left panel). However, luteinized granulosa cells secreted little to no LIF, and culturing in the presence of 1% O2 did not affect LIF secretion (Figure 3A, right panel). Whereas, both non-luteinized and luteinized granulosa cells secreted VEGF in response to low oxygen, hCG, and low oxygen in combination with hCG synergistically increased VEGF secretion (Figure 3B). Granulosa cell secretion of progesterone was hCG-dependent in both non-luteinized and luteinized granulosa cells, though non-luteinized granulosa cells secreted less hCG-stimulated progesterone in 1% O2 versus 20% O2. Granulosa cell expression of KDM3A was used to assess appropriate hypoxia signaling. Luteinized granulosa cells had increased KDM2A expression when cultured in a 1% O2 environment. Interestingly though, non-luteinized granulosa cells had variable changes in KDM2A expression, with two animals’ KDM3A expression decreasing when cultured in 1% O2 and two animals’ KDM3A expression increasing.

## LIF secretion by human granulosa cell line, KGN

The human granulosa cell line, KGN, secreted more LIF in response to forskolin (a protein kinase A activator, because KGN cells lack LHCGR and FSHR). Addtionally, KGN cells secreted more LIF in the presence of 1% O2 compared to 20% O2, and low oxygen and forskolin synergistically increased LIF secretion. KGN secretion of progesterone was also stimulated by forskolin, low oxygen and synergistically by the combination of forskolin and low oxygen.

## Regulation of granulosa cell function by LIF

Luteinized granulosa cells secreted more VEGF after treatment with LIF or hCG, but LIF and hCG did not synergistically increase VEGF secretion. Granulosa cell secretion of progesterone (both basal and hCG-stimulated) were unaffected by treatment with LIF. Granulosa cell expression of SOCS3 was used to assess appropriate LIF signaling, and SOCS3 expression increased after treatment with LIF, though there was variability in the fold change seen between animals (4-fold to 12-fold).

# Discussion

Rhesus granulosa cells are responsible for the secretion of LIF into follicular fluid seen after induction of ovulation. Non-luteinized granulosa cells of rhesus ovaries increase secretion of LIF in response to both gonadotropins (hCG and FSH) and low oxygen. Interestingly, the secretion of LIF by rhesus granulosa cells appears to be dependent on the stage of luteinization, since only non-luteinized granulosa cells secreted LIF and increased secretion based on gonadotropins and oxygen levels. Whereas, luteinized granulosa cells secreted little to no LIF, and secretion could not be enhanced by either gonadotropins or oxygen levels. Since KGN cells also secrete LIF in a PKA and oxygen dependent manner, it is likely that regulation of LIF secretion s conserved between rhesus macaques and humans. Although luteinized granulosa cells did not secrete LIF, these luteinized granulosa cells responded to LIF treatment thereby indicating that LIF likely acts as a autocrine/paracrine factor within the follicle to regulate ovulatory processes.

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# Conflict of interest

Provide a statement indicating any potential or actual conflicts of interest with respect to the work reported in the article.

# Author contributions

Indicate the contributions to the manuscript made by each author, identified by initials corresponding to first and last names.

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# Tables

Table 1 – qPCR Primers and Probes

# Supplemental Information

## Materials and Methods

### Controlled Ovulation (COv)

Rhesus macaque females (n = 12) were monitored for initiation of menstruation to identify the beginning of the follicular phase (Cycle Day 1). Four to six days later (depending on length of previous menstrual cycle), serum E2 and P levels were monitored daily 1 and COv protocols were initiated when serum E2 levels reached 90–120 pg/ml, indicating selection of the dominant antral follicle; see 1 for details of the rhesus macaque COv protocol). The ovulatory hCG bolus was administered 20–22 h postintrafollicular injection, to ensure adequate production of shRNAs prior to the increase in PGR mRNA that typically occurs within 12 h post-hCG delivery. Ovaries were evaluated by laparoscopic methods for ovulation 96 h after intrafollicular vector injection, and several (n = 4/vector) were collected for histological evaluation and immunohistochemistry to observe follicular morphology and PGR protein expression as described below. Daily serum samples were collected for E2 and P analyses throughout COv protocols until time of ovariectomy. From 3

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### Controlled Ovarian Stimulation

All experiments involving rhesus females were performed with approval of the Oregon Health & Science University Institutional Animal Care and Use Committee at the Oregon National Primate Research Center (ONPRC), in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rhesus monkeys were cared for by the ONPRC Division of Comparative Medicine (DCM). The animals were housed in individual cages with auditory, visual, and olfactory interaction with male and female conspecifics in a temperature controlled environment (24 °C) under a fixed 12L:12D photoperiod (lights on from 700 h to 1900 h) with ad libitum access to drinking water. Individuals were cared for by the Oregon National Primate Research Center in accord with the National Research Council’s Guide for the Care and Use of Laboratory Animals (National Research Council of the National Academies, 2011), which included daily health checks to ensure normal behavior, food consumption, and waste production. Additionally, routine physical examinations, hematological studies, fecal parasite checks, tuberculin testing, and dental cleaning were performed periodically. All surgical procedures were performed by the veterinary staff of the ONPRC DCM Surgical Services Unit (SSU).

NLGCs were collected from female monkeys undergoing COS cycles to induce multifollicular development prior to an ovulatory stimulus. Briefly, females were monitored daily for onset of menstruation, and within the first 3 days were placed on an injection regimen consisting of 6 days of recombinant human FSH (20 IU, i.m.) at 0800 and 1600 h, followed by 1 day of FSH:recombinant human LH (20 & 30 IU each, i.m.) at 0800 and 1600 h with the GnRH antagonist acyline (75 lg/kg, s.c.) at 0800 h. The following morning, follicular contents were aspirated by laparoscopic techniques (Wolf et al., 1989) to retrieve immature germinal vesicle (GV)-stage cumulus oocyte complexes and NLGCs. Aspirates from individual females were prepared for separate experiments

National Research Council of the National Academies. (2011). *Guide for the Care and Use of Laboratory Animals*. *Guide for the Care and Use of Laboratory Animals* (8th ed.). Washington, D.C.: National Academies Press. https://doi.org/10.17226/12910

Wolf, D. P., Vandevoort, C. A., Meyer-Haas, G. R., Zelinski-Wooten, M. B., Hess, D. L., Baughman, W. L., & Stouffer, R. L. (1989). In vitro fertilization and embryo transfer in the rhesus monkey. *Biology of Reproduction*, *41*(2), 335–346. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list\_uids=2508776

### Full Data File

### R Code

### Granulosa cell isolation

Oocytes were removed from the follicular aspirates, and remaining cells were pelleted by centrifugation at 170 x g (4 °C). Pelleted cells were resuspended in Ham F10 medium containing 25 mM HEPES and 0.1% BSA (pH 7.4). Red blood cell contaminates were removed by Percoll (Sigma- Aldrich) gradient centrifugation (30% Percoll/Hanks basal salt solution containing 0.1% BSA without phenol red; pH 7.4) at 500 x g for 30 min (4 °C). The granulosa cell (GC) fraction was then isolated and diluted 1:5 in Ham F-10/0.1% BSA medium to remove any contaminating Percoll. A final centrifugation was performed at 170 x g to pellet GCs.

### RNAScope

Ovaries were taken from Rhesus Macaques before (0 hour) and subsequent (12, 24 and 36 hours) to an injection of an ovulatory administration of hCG. The ovaries were obtained laparoscopically and were fixed in 10% neutral-buffered formalin overnight, dehydrated in a series of ethanol solutions (50, 70, and 100%) and embedded in paraffin. The paraffin-embedded tissues were serially sectioned at 5 μm using an American Optical (Southbridge, MA) microtome and mounted on Superfrost slides (Fisher, Santa Clara, CA) in the Imaging & Morphology Core Laboratory at ONPRC. Detection of LIF mRNA was obtained as previously described1. The LIF target, positive and negative control targets (cyclophilin B and bacterial dihydrodipicolinate reductase, respectfully), preamplifier, amplifier, label probe, protease inhibitor, hybridization buffers A, B and C, and wash buffer were all from Advanced Cell Diagnostics (Hayward, CA). After staining and dehydrating the slides were mounted using DPX mountant for histology (Sigma-Aldrich, St. Louis, MO). Slides were imaged using a 40x objection on an Olympus BX40 scope with an Olympus DP72 camera.

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