ORIGINAL ARTICLES

Using Patient-Derived Granulosa Cell Organoids as A Model System for Studying Granulosa Cell Dysfunction in Patients with Polycystic Ovary Syndrome (PCOS)

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Objective: *To determine the feasibility of creating organoids from granulosa cells obtained at the time of oocyte retrieval and use these as a model system to investigate granulosa cell dysfunction in patients with polycystic ovary syndrome (PCOS).*

Design: *Laboratory based study or prospective experimental study*

Materials and methods: *Follicular fluid was collected from 44 patients at the time of oocyte retrieval. Granulosa cell organoids were created and used to examine granulosa cell dysfunction in patients with PCOS.*

Main Outcome Measures: *The main outcome measure was the ability to grow granulosa cell organoids from granulosa cells obtained at the time of oocyte retrieval. Secondary outcome measures included growth of organoids, morphology, and defining the differentially expressed genes (DEGs) by bulk-RNA sequencing in patients with and without PCOS.*

Results: *Granulosa cell organoids can be produced from granulosa cells obtained from the follicular fluid at the time of oocyte retrieval with a growth rate of 100% (44 patients). The presence of granulosa cells was confirmed by hematoxylin and eosin stains and confirmed by pathology. The morphology of granulosa cell organoids was examined by light microscopy and revealed a spherical pattern demonstrating 3D shape. Bulk RNA-sequencing was used to determine differences in gene expression in granulosa cells of patients with and without PCOS. Patients with PCOS had upregulated DEGs in TGF-B signaling, steroid metabolism, apoptosis and NOTCH signaling, and downregulation in luteinization, calcium channel and FGFR signaling.*

Conclusion: *We report a novel protocol for development of granulosa cell organoids created from follicular fluid obtained at the time of oocyte retrieval. Significant differences in gene expression are shown from granulosa cell organoids between patients with and without PCOS in relation to apoptosis, folliculogenesis, and steroid metabolism. Granulosa cell organoids represent a novel experimental model system to gain more insight into the pathophysiology of PCOS.*

BACKGROUND

Granulosa cells are somatic cells that surround the oocyte within a growing ovarian follicle. They are involved in complex, bi-directional crosstalk via gap junctions. A functional line of communication Between the granulosa cells and the oocyte serve to coordinate follicular growth, oocyte maturation and ovulation, and steroidogenesis. Granulosa cells produce estrogen and Anti-Müllerian hormone (AMH)1,2. Estrogen production is crucial in regulating the menstrual cycle, thickening the

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Financial Disclosures: None.
0024-7758

endometrium for implantation, and maintaining adequate reproductive health. Granulosa cell dysfunction represents a key part of the pathophysiology of polycystic ovarian syndrome (PCOS)

Patients with polycystic ovary syndrome (PCOS) have known alterations in function and gene expression which can lead to infertility and hormonal imbalances. PCOS is a common endocrine disorder affecting 10-15% of reproductive aged females [4]. PCOS has many clinical implications such as ovulatory dysfunction resulting in irregular menses, hyperandrogenism, and polycystic appearing ovaries on ultrasound. Women with PCOS suffer from infertility and metabolic disturbances such as insulin resistance, dyslipidemia, and hypertension[5]. Because of these metabolic disturbances, patients with PCOS are at risk for diabetes mellitus type 2 and cardiovascular disease.[6]

Granulosa cell dysfunction manifests in multiples ways in women with PCOS. Women with PCOS have an increased number of granulosa cells residing in their follicles due to increased number of antral follicles [7]. This is thought to be due to arrested follicular growth from decreased inhibin A and B leading to a buildup of excess follicles[8]. Additionally, granulosa cells in women with PCOS have altered hormonal production, which is responsible for infertility and clinical signs of androgen excess [9]. Some women with PCOS may also have insulin resistance which directly affects the mechanism in which granulosa cells produce estrogen [10]. Finally, increased oxidative stress and chronic low-level inflammation affects granulosa cell function and can worsen the sequelae of PCOS. [11,12].

Patients with PCOS demonstrate alterations in granulosa cell gene expression. Prior investigations have found abnormal mRNA expression among several members of the IGF, EGR, and TNF families' families in granulosa cells from women with PCOS, as well as altered estrogen receptor signaling [13]. A study evaluating cell survival and proliferation rates in the granulosa cells of patients with PCOS found lower apoptotic rates compared to patients without PCOS[14], which is in line with the increased granulosa cell proliferation seen in patients with PCOS[15]. In addition, patients with PCOS have altered gene expression of steroidogenic enzymes such as aromatase and steroidogenic acute regulatory protein (STAR), which leads to a relative decrease in estrogen production compared to androgens[16]. Granulosa cells from patients with PCOS demonstrate increased reactive oxygen species [11], produce more AMH, and have a greater number of follicles stimulating hormone receptors (FSH R), which may contribute to deficits in oocyte maturation[17].

There are no studies to date that have described the creation of granulosa cell organoids derived from primary granulosa cells obtained at the time of oocyte retrieval during an in vitro fertilization (IVF) cycle or planned oocyte cryopreservation cycle. Organoids are three-dimensional (3D) structures grown in a laboratory dish from primary cells that recapitulate the functional and phenotypic features of their tissue of origin. The purpose of this study is to show that granulosa cells obtained at the time of oocyte retrieval can be maintained as three-dimensional organoids and serve as a model system for investigating the pathophysiology of PCOS from a structural, functional, and gene expression level.

MATERIALS AND METHODS

Collection Of Granulosa Cells

This study was approved by the Institutional Review Board (IRB) of Northwell Health (IRB#22-0217). Study protocols were reviewed and approved by Northwell Health and Cold Spring Harbor Laboratory, and written consent was obtained from all patients in this study. Table 1 displays the demographic and baseline characteristics for all patients. Baseline characteristics such as age, body mass index (BMI), antral follicle count (AFC), Anti-Müllerian hormone (AMH), estradiol levels on day of oocyte maturation trigger, number of follicles visualized on ultrasound, total number of oocytes retrieved, and total number of metaphase II oocytes (MIIs) were collected. Patients with PCOS were

Table 1. Demographic and baseline characteristics of study participants

	PCOS (n = 20)	Control (n = 24)	p value
Age (years)	35.1 ± 4.8	34.1 ± 5.6	0.59
BMI (kg/m 2)	29.9 ± 7.2	24.6 ± 3.6	0.01
Antral follicle count (n)	22.5 ± 10.3	14.6 ± 9.1	0.003
AMH (ng/mL)	7.4 ± 4.1	3.4 ± 1.9	0.003
# of follicles at trigger	24.2 ± 10.5	17.5 ± 7.8	0.003
Estradiol on day of trigger (pg/mL)	3524 ± 1250	2722 ± 1256	0.04
# of oocytes retrieved (n)	24.75 ± 13.6	18.6 ± 9.1	0.12
# of metaphase II oocytes (n)	17.0 ± 9.6	14.4 ± 7.4	0.04

diagnosed based on the modified Rotterdam criteria of at least 2 of the following: oligo or anovulation, clinical or biochemical signs of hyperandrogenism, and polycystic ovaries (≥ 20 follicles per ovary on either ovary or an ovarian volume > 10 cm3 in at least one ovary) [18]. Patients in the control group had one of the following diagnoses: unexplained infertility, planned oocyte or embryo cryopreservation, male factor infertility, tubal factor infertility, or recurrent pregnancy loss.

Supplemental figure 1 displays the experimental schematic. Patients underwent controlled ovarian stimulation with gonadotropins based on one of the clinic's standard protocols and were triggered with either human chorionic gonadotropin (hCG), leuprolide acetate, or a combination of both to achieve final oocyte maturation. Each patient was taken to the operating room 35 hours after the trigger shot for an ultrasound guided transvaginal oocyte retrieval under intravenous sedation. Follicular fluid containing oocytes was aspirated into a Falcon 14ml test tube (Corning, Cat#352057) and brought to the embryology laboratory where the follicular fluid was examined for the cumulus oocyte complex (COC). The COCs were pooled into a petri dish in follicular fluid and culture media Multipurpose Handling Medium-Complete (MHM-C) with Gentamicin (Fujiform, Cat#90166-500). Upon identification of the oocyte, the embryologist mechanically removed the cumulus granulosa cells from the oocyte with a pipette tip. The granulosa cells were collected into a CryoTubeTM Vial (Thermo Scientific) and transported to Cold Spring Harbor Laboratory on ice.

Generation Of Organoid Cultures from Granulosa Cells

Granulosa cells collected from follicular aspirate were first washed with Dulbecco's Modified Eagle Medium DMEM (Gibco, Cat# 11965-092) before a 10-minute incubation period in ACK Lysis Buffer (Homemade) at 4C. The cell suspension was then pelleted with a 5min, 300g spin at 4C. The subsequent cell pellet was mixed into a 70/30 Matrigel (Corning, Cat# 356231) and culture medium mixture with a density of 500 cells per microliter. Droplets were placed on a 6-well culture plate and allowed to polymerize at 37C for 10 minutes before adding culture medium to each well. Based on previous studies by Kossowska-Tomaszczuk et al [17], the culture medium was optimized using High-Glucose DMEM (Gibco, Cat# 11965-092), 15% Fetal Bovine Serum (Corning, Cat# 35-010-CV), 1X Pen/Strep (ThermoFisher, Cat# 15070063), 100ng/ml Recombinant Follicle Stimulating Hormone (Sigma-Aldrich, Cat# F4021), and 1000IU/mL Leukemia Inhibiting Factor (Peprotech, Cat# 300-05).

Organoid Maintenance

Media refreshment occurred every four to five days. Organoid lines were passaged at 14-28 days old, depending on their growth rates. Matrigel droplets were lifted from their wells and placed into Cell Recovery Solution (Corning, Cat# 354253) for one hour at 4C. Following centrifugation, the pellet was

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Passaging Of Granulosa Cell Organoids

Once the organoids were 28 days old and deemed ready for passage, they were spun down at 300g. The cell pellet was resuspended in either TryplE Express (ThermoFisher, Cat# 12604039), Gentle Cell Dissociation Reagent (Stem Cell Technologies, Cat# 100-0485), or a 2mg/mL Collagenase (Sigma Aldrich, Catt# C9407) solution. Digestion occurred at 37C for 5-10 minutes for TryplE, 25C for 10-15 minutes for Gentle Cell Dissociation Reagent, or 37C for 30-60 minutes for Collagenase. Brief mechanical trituration via pipetting was performed at the end of the incubation period before centrifugation.

Creation Of Slides For H&E

After organoids were embedded in paraffin, serial cuts were performed to use for hematoxylin and eosin staining (H&E).

Rna Isolation

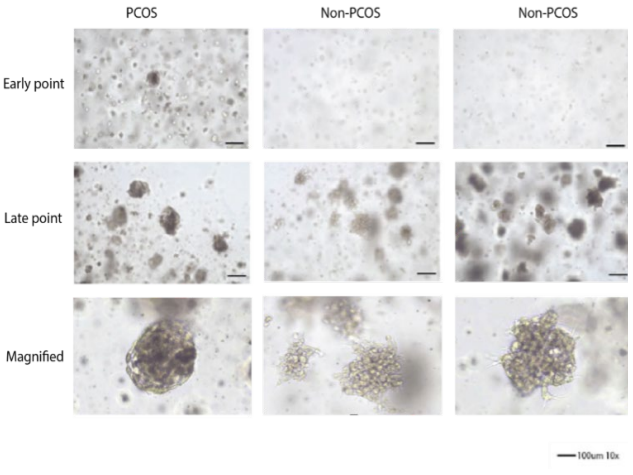
Matrigel droplets containing the selected organoids were lifted from their wells and placed into Cell Recovery Solution (Corning, Cat# 354253) for one hour at 4C. The organoids were then spun down at 300g. The cell pellet was resuspended in 400uL of Ambion TRIzol Reagent (Thermo Scientific, Cat #15596018) and mixed to promote cell lysis. RNA was extracted from control and Polycystic Ovary Syndrome (PCOS) organoid lines using the Zymo Direct-zol RNA Microprep Kit (Zymo, Cat# R2062), following the manufacturer's protocol and specifications. Total RNA was isolated from P0 of granulosa cell organoids from patients with and without PCOS using Zymo RNA isolation kit (R2062) according to manufacturer's instructions. Starting from a total 250 ng RNA, rRNA depletion protocol was followed according to suggested guidelines from the manufacturer. Strand specific RNA seq libraries were prepared using NebNext Ultra II kit (E7760L) and sequenced on Illumina NextSeq.

RESULTS AND DISCUSSION

Organoid Formation

Our method of organoid formation proved to be successful in culturing and creating three dimensional organoids from single cells. Granulosa cells obtained at the time of oocyte retrieval can self-organize into a 3D sphere and propagate through one passage. The media was adapted from Kossowska-Tomaszczuk et al [19] which contained media containing leukemia inhibiting factor (LIF), Follicle Stimulating Hormone (FSH), high glucose DMEM, and FBS. The media was refreshed every 4-5 days and TRIzol was collected for RNA extraction. Light microscopy images were taken at the beginning of organoid formation, around 14 days after initial plating, and prior to passaging as shown in Figure 1.

Figure 1. Row 1 shows Granulosa cell organoids from the original plating. The granulosa cells form spherical structures which can be morphologically assessed. Row 2 shows granulosa cell organoids at a later point in the original passage. Row 3 shows a magnified view of the organoids demonstrating a spherical structure.

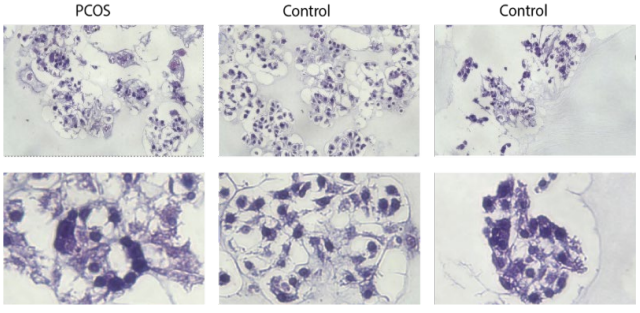


Organoid Morphology

Granulosa cell organoid morphology was assessed via light microscopy as shown in Figure 1. The granulosa cell organoids show spherical structures, highlighting their 3-dimensional nature. Figure 1 shows 1 PCOS sample and 2 control samples. The top row shows organoids from the early stage, while the second row displays organoids from a later stage of the original passage. The bottom row displays a magnified view of the later stage organoids.

We identified granulosa cells within the sections of granulosa cell organoids that were stained with hematoxylin and eosin (H&E) [Fig. 2]. The organoids present a spherical structure and characteristic appearance of granulosa cells, as confirmed by a pathologist.

Figure 2. Hematoxylin and eosin (H&E) staining of the samples from the pilot study. The top row shows a spherical structure of the organoids and the bottom row shows a magnified view of the granulosa cells displaying their characteristic appearance. Cellular structure confirmed by pathology.



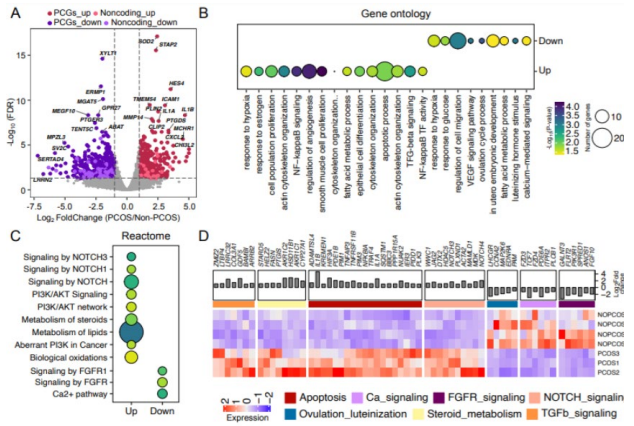
Bulk RNA-sequencing

Altered gene expression in granulosa cells from patients with PCOS can shed some insight into the pathophysiology of this disorder and serve as a basis for therapeutic agents in the management of this disease. Granulosa cells are involved in folliculogenesis which is the development of ovarian follicles. These follicles change throughout the process of oocyte maturation. In PCOS, the altered gene expression in granulosa cells is often related to steroidogenesis, insulin signaling, and inflammation. Previous studies have found abnormal expression of genes related to ovarian steroidogenesis, fatty acid biosynthesis and lipid metabolism[20]. No studies to date have created granulosa cell organoids from granulosa cells obtained at the time of oocyte retrieval and studied differences in gene expression between patients with PCOS and a non-PCOS control group. Patterns of differentially expressed genes (DEGs) in a hierarchal clustering are shown in Figure 4.

In our study, granulosa cells from patients with PCOS exhibited upregulation of genes in several pathways including NOTCH pathways, PI3K/AKT network, metabolism of steroids and lipids and oxidative metabolism. In contrast, genes involved in the FGFR and calcium channel pathways were downregulated in patients with PCOS [Fig. 3C]. Figure 3D displays upregulated genes in PCOS that are involved in TGF-B signaling, steroid metabolism, apoptosis and NOTCH signaling, where there is downregulation in luteinization, calcium channel and FGFR signaling.

Figure 3: PCOS deregulated genes and associated pathways.
A. Volcano plot showing differentially expressed genes between PCOS and non-PCOS transcriptome data. Red (Darker) and Purple (Darker) color denotes significantly up- and down-regulated protein coding genes respectively. The lighter color indicates significantly deregulated noncoding RNAs. The vertical dotted lines indicate log2 fold change cut-off (+1 and -1) and the horizontal dotted lines indicate -log10 adjusted P-value < 0.05. B. Biological processes associated with up- and down-regulated genes in PCOS. Significant terms are selected with p-value < 0.05. Size of circles indicates the number of differentially expressed genes involved in the biological process and the color gradient indicates level of significance. All the statistics for biological processes are derived using GeneSCF with gene ontology database. C. Molecular pathways associated with up- and down-regulated genes in PCOS. Significant pathway terms are selected with p-value < 0.05. Size of circles indicates number of differentially expressed genes involved in the biological process and the color gradient indicates level of significance. All the statistics for pathways are derived using

GeneSCF with reactome database. D. Heatmap shows expression of genes from key signaling pathways associated with PCOS. Upper bar plot indicates log2fold change (positive: Upregulated in PCOS and negative: Downregulated in PCOS). All the genes displayed in heatmap were selected based on adjusted p-value < 0.05.



It is well known that there are increased inflammatory markers in patients with PCOS. In our study, there was upregulation of genes involved in the PI3K/AKT pathway and NFkB pathway. Our findings suggest that dysregulation in these pathways may play a role in the pathogenesis of PCOS. Studies have shown that there is an increased rate of apoptosis in granulosa cells of women with PCOS in the PI3/AKT pathway via regulation of USP25 and PTEN [21]. This accelerated follicular death disrupts follicular development and contributes to infertility.

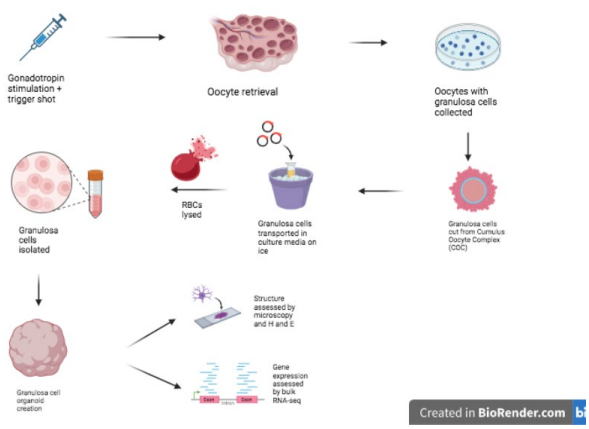
Steroid metabolism is altered in PCOS which is evident in the classic phenotype of women with PCOS displaying increased ovarian androgen production. Several genes involved in the steroid metabolism pathway were upregulated in PCOS as shown in Figures 3B and 3C. Other studies have previously found aberrant control of genes involved in steroid metabolism in granulosa cells from PCOS[22].

In the current study, genes involved in the FGFR1 pathway were downregulated. Fibroblast growth factors are important mediators in several cellular processes such as cell survival, proliferation and steroidogenesis [23].

Genes involved in the calcium signaling pathway were also downregulated as shown in Figures 3C and 3D. This pathway is involved in oocyte maturation and development through paracrine signaling which is altered in patients with PCOS. A previous study found alterations in an important mediator, CASR, in patients with PCOS [24].

Previous studies have also found upregulation in PCOS in the NOTCH pathways, which play a role in steroidogenesis. Specifically, altered NOTCH 2 can lead to altered cumulus oocyte expansion resulting in dysfunction of folliculogenesis [25].

Figure 4. Schematic of the study. Granulosa cells were collected during oocyte retrieval and transported to the laboratory on ice in culture media. Granulosa cells were isolated using ACK buffer to lyse red blood cells. Organoids were created using Matrigel as an extracellular matrix. Microscopy, H and E staining and bulk RNA-sequencing were performed on organoids. Created with BioRender.com.



Study Strengths and Limitations

Strengths of this study include the description of the novel technique for creating granulosa cell organoids using granulosa cells obtained at the time of oocyte retrieval. This is the first study of its kind to use

granulosa cells in a three-dimensional context to create a model system for examining ovarian biology and disorders of reproduction in vitro.

Limitations to the study include the fragility of organoids that limits their propagation in long-term passages. There is room for further optimization of the growth media to enhance propagation of organoid passaging and examine secretion of growth factors to gain insight in paracrine signaling.

CONCLUSIONS

Polycystic ovary syndrome is a complex endocrine disorder that contributes to infertility. It is multifactorial in nature and much of its pathophysiology lies within granulosa cell dysfunction.

This study describes a new experimental model for elucidating the pathophysiology of polycystic ovary syndrome as it relates to granulosa cell structure, function, and gene expression. This is the first study to describe the creation of granulosa cell organoids from follicular fluid obtained at the time of oocyte retrieval. Our lab documented a successful method of culturing granulosa cells in a specialized environment that encourages organization and growth into three-dimensional structures.

While this model is in its early experimental phase, it serves as a pilot for future studies on altered granulosa cell function in patients with polycystic ovary syndrome. This organoid model also provides a valuable tool for investigating the structure, function, and disorders related to ovarian biology, folliculogenesis, and steroidogenesis. Potential applications for this model exist in reproductive medicine as it relates to endocrine disorders, and the model could be used to correlate in vitro fertilization outcomes with granulosa cell health.

Granulosa cell organoids are an emerging technology with the potential to improve reproductive outcomes in patients with PCOS undergoing in vitro fertilization.

DECLARATIONS

Conflict Of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgements

The authors acknowledge the patients and embryology department at Northwell Health Fertility for their assistance in obtaining granulosa cells during oocyte retrievals and the Beyaz Lab at Cold Spring Harbor Laboratory for their assistance with this project. We thank Cold Spring Harbor Laboratory Cancer Center Shared Resources (Sequencing, Organoid and Histology Core Facilities) supported in part by the National Cancer Institute Cancer Center Support Grant 5P30CA045508. This work was financially supported by grants to

S.B from the Oliver S. and Jennie R. Donaldson Charitable Trust, Chan Zuckerberg Initiative/Silicon Valley Community Foundation (2021-239862), the Cold Spring Harbor Laboratory and Northwell Health Affiliation. We also thank Northwell Health Department of OBGYN for supporting this work.

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