Human induced pluripotent stem cell-derived ovarian support cell co-culture improves oocyte maturation *in vitro* after abbreviated gonadotropin stimulation

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Abstract

Assisted reproductive technologies (ART) have significantly impacted fertility treatment worldwide through innovations such as *in vitro* fertilization (IVF) and *in vitro* maturation (IVM). IVM holds promise as a technology for fertility treatment in women who cannot or do not wish to undergo conventional controlled ovarian hyperstimulation (COH). However, IVM has historically shown highly variable performance in maturing oocytes and generating oocytes with strong developmental capacity. Furthermore, novel IVM approaches are usually highly limited to use in cycles lacking human chorionic gonadotropin (hCG) triggers, which is not standard practice in fertility treatment. We recently reported the development of ovarian support cells (OSCs) generated from human induced pluripotent stem cells (hiPSCs) that recapitulate dynamic ovarian function *in vitro*. Here we investigate the potential of these OSCs in an IVM co-culture system to improve the maturation of human cumulus-enclosed immature oocytes retrieved from abbreviated gonadotropin stimulated cycles. We reveal that OSC-IVM significantly improves maturation rates compared to existing IVM systems. Most importantly, we demonstrate that OSC-assisted IVM oocytes are capable of robust euploid blastocyst formation, a key marker of their clinical utility. Together, these findings demonstrate a novel approach to IVM with broad applicability to modern ART practice.

Introduction

It is estimated that nearly 1 in 8 women in the United States and many Western nations have sought fertility treatment.[1] Assisted reproductive technologies (ART) such as *in vitro* fertilization (IVF) offer remarkable benefits for treating aspects of infertility for certain patient populations.[2,3] However, the use of high doses of gonadotropin in stimulation regimens has resulted in uncomfortable and sometimes serious and long lasting side effects for women undergoing the process, with rising costs continuing to limit access in many nations.[4,5] In addition, for some women for whom conventional controlled ovarian hyperstimulation is contraindicated, standard IVF practice is not feasible due to the medical risk it poses.[6–8] Therefore, new technologies that allow for a reduction of gonadotropin utilization in fertility treatments are important to potentially improve patient outcomes and ART accessibility.

One option for reducing gonadotropin usage in fertility treatment is through the application of minimal or abbreviated stimulation cycles. Such cycles drastically reduce the cost and complications of IVF, while nearly completely eliminating the chance for severe complications such as ovarian hyperstimulation syndrome (OHSS).[9–11] Depending on the protocol, abbreviated gonadotropin stimulation results in a cohort of oocytes that are mostly or all immature, including germinal vesicle containing (GV) oocytes and oocytes lacking a germinal vesicle and polar body (metaphase I, MI), while some regimens may yield a small pool of mature oocytes with a polar body (metaphase II, MII).[11–13] In abbreviated gonadotropin cycles, *in vitro* maturation (IVM) holds promise as an ART strategy to mature oocytes outside of the body for use in fertility treatments. Numerous studies have employed IVM effectively, often through the application of reproductive cell culture media supplemented with growth factors, small molecules, and gonadotropins.[11,13–26] However, many of these studies have shown mixed results of IVM applications in terms of oocyte maturation rate, cleaved embryo formation, and blastocyst formation with some studies showing lower pregnancy rates from IVM cycles. Therefore, improvement is needed in IVM strategies that yield robust, high-quality oocytes with strong developmental competence.

Oocyte maturation is a coordinated series of molecular processes occurring within the oocyte nucleus and cytoplasm.[27,28] These molecular processes result in both nuclear maturation, namely extrusion of the first polar body (PB1), assembly of the second meiotic spindle, and cytoplasmic maturation, defined as distribution of organelles in the oocyte cytoplasm and deposition of proteins and transcripts needed for fertilization competence and subsequent embryogenesis.[29] Oocyte maturation normally occurs within the ovary in response to complex, temporally-regulated, extrinsic signals provided through hormone signaling, growth factor production, and nutrient dynamics in the follicular environment.[28,30–36] Many of these processes are regulated by somatic ovarian cells, such as granulosa cells, theca cells, and stroma cells. Limited research has been performed to assess the use of these somatic cell types for *in vitro* maturation, but evidence exists for their utility in human IVM applications.[37–40] However, the use of patient-derived somatic cell types besides cumulus cells is challenging due to inconsistent cell type composition retrieval, difficulty in extracting and purifying populations, and infeasibility of extracting the needed quantities of support cells without harming patients. In addition, the issue underlying some patients' infertility could reside in inappropriate signaling within and among their own follicular cells. Therefore, new IVM systems that effectively and consistently deliver specific ovarian support cell types for co-culture with immature oocytes is a salient potential platform for human oocyte IVM.

Our previous work described the generation of ovarian support cells (OSCs) from human induced pluripotent stem cells (hiPSCS) in a rapid, efficient, and reproducible manner through transcription factor (TF)-directed differentiation.[41] The OSCs primarily are composed of FOXL2+ AMHR2+ granulosa-like cells as well as ovarian NR2F2+ stroma-like cells. The OSCs are steroidogenic, producing aromatase in response to follicle stimulating hormone (FSH) stimulation to catalyze the estradiol synthesis pathway,

and produce the necessary growth factors needed for robust paracrine interaction with oocytes and cumulus cells. In this study, we investigated the potential of hiPSC-derived OSCs to improve *in vitro* maturation of oocytes in a co-culture system with immature cumulus oocyte complexes (COCs) retrieved from subjects following abbreviated gonadotropin cycles.

Materials and Methods

Collection of Cumulus Oocyte Complexes (COCs)

Subject ages, IRB and Informed Consent

Subjects were enrolled in the study through Ruber Clinic (Madrid, Spain), Spring Fertility Clinic (New York, USA) and Pranor Clinic (Lima, Peru) using informed consent (CNRHA 47/428973.9/22, IRB # 20225832, Western IRB, and Protocol #GC-MSP-01 respectively). Subject ages ranged between 19 and 37 years of age with an average of 28. Oocytes retrieved from the Ruber and Pranor clinics were utilized for maturation analysis endpoints only, while oocytes retrieved from Spring Fertility were utilized for embryo formation endpoints.

Stimulation characteristics

25 subjects received three to four days of stimulation using 325-600 IU of rFSH with an hCG trigger in preparation for immature oocyte aspiration for Experiment 1, with an AMH value of >1ng/ml (see below). 21 subjects received three consecutive days of 200 IU of rFSH with an hCG trigger in preparation for immature oocyte aspiration for Experiment 2, with an AMH value of >1.5ng/ml used as an inclusion criterion to enrich for donors yielding more oocytes (see below). Seven subjects received five doses of clomiphene citrate (Clomid) (100 mg) with an additional one to two doses of 150 IU rFSH with or without an hCG trigger for Experiment 2 with the goal of subsequent embryo formation, and an AMH value of >2.0ng/ml utilized as an inclusion criterion (see below). Gonadotropin injections were initiated on day 2 of a natural menstrual cycle or on the fifth day following cessation of oral contraceptive pills. A complete table of donor stimulation regimens for each donor in the study is provided in Supplementary Table 1.

Aspiration of small ovarian follicles to retrieve immature cumulus oocyte complexes

Aspirations were performed 36 hours after the trigger injection (10,000 IU hCG) using a transvaginal ultrasound with a needle guide on the probe to retrieve oocytes for co-culture experiments. Aspiration was performed using ASP medium (Vitrolife) without follicular flushing using double lumen 19-gauge needles (double lumen needles were selected due to the additional stiffness provided by the second channel inside the needle). Vacuum pump suction (100 mm Hg) was used to harvest follicular contents through the aspiration needle and tubing into a 15 ml round bottom polystyrene centrifuge tube. For the conditions where the final outcome was embryo formation, aspirations were performed 36 hours after trigger injection (2,500 IU hCG) or 48 hours after last rFSH injection for untriggered cycles. Aspiration was performed without follicular flushing using a single lumen 19- or 20-gauge needle with a vacuum pump suction (~200 mm Hg) used to harvest follicular contents through the aspiration needle and tubing into a 15 ml round bottom polystyrene centrifuge tube. In all cases, rapid rotation of the aspiration needle around its long axis, when the follicle had collapsed, provided a curettage effect to assist the release of COCs into the aspirate fluid. Although follicles were not flushed, the aspiration needle was removed from the subject and flushed frequently throughout the oocyte retrieval procedure to limit clotting and needle blockages.

Follicular aspirates were examined in the laboratory using a dissecting microscope. Aspirates tended to include more blood than typical IVF follicle aspirations, so samples and needles were washed with HEPES media (G-MOPS Plus, Vitrolife) to minimize clotting. Often, the aspirate was additionally filtered

using a 70-micron cell strainer (Falcon, Corning) to improve the oocyte search process. COCs were transferred using a sterile Pasteur pipette to a dish containing LAG Medium (Medicult, Cooper Surgical) until use in the IVM procedure. The number of COCs aspirated was equal to roughly 40% of the antral follicles seen in the subject's ovaries on the day of harvest.

Preparation of Ovarian Support Cells (OSCs)

OSCs were created from human induced pluripotent stem cells (hiPSCs) according to transcription factor (TF)-directed protocols described previously.[41] The OSCs were produced in multiple batches and cryopreserved in vials of 120,000 to 150,000 live cells each and stored in liquid nitrogen in CryoStor CS10 Cell Freezing Medium (StemCell Technologies).

Culture dishes (4+8 Well Dishes, BIRR) for oocyte maturation experiments were prepared with culture media and additional constituents in 100µl droplets under mineral oil the day before oocyte collection, and equilibrated in the incubator overnight. The morning of oocyte collection, cryopreserved OSCs were thawed for 2-3 minutes at 37ºC (in a heated bead or water bath), resuspended in OSC-IVM medium and washed twice using centrifugation and pelleting to remove residual cryoprotectant. Equilibrated OSC-IVM media was used for final cell resuspension. OSCs were then plated at a concentration of 100,000 OSCs per 100µl droplet by replacing 50µl of the droplet with 50µl of the OSC suspension 2-4 hours before the addition of oocytes to allow for culture equilibration and media conditioning (Figure 1A).

***In vitro* maturation**

COCs were maintained in preincubation LAG Medium (Medicult, Cooper Surgical) at 37ºC for 2-3 hours after retrieval prior to introduction to *in vitro* maturation conditions.

Two sets of experimental comparisons were performed to address the following goals:

Experiment 1 (OSC activity): The purpose of this comparison was to determine whether the stimulated OSCs were the active ingredient of the co-culture system. For this purpose, media in experimental and control conditions were prepared following the manufacturer's recommendations (Medicult, Cooper Surgical), and further supplemented with androstenedione and doxycycline (both necessary for activation of OSCs) in order to compare maturation outcomes with or without OSCs in the same medium formulation (see Table 1 below).

Experiment 2 (OSC clinical relevance): The purpose of this experiment was to compare the efficacy of the OSC-IVM system and the commercially available *in vitro* maturation system (Medicult IVM). For this purpose, the Control Group condition was prepared and supplemented by following the manufacturer's recommendations (Medicult, Cooper Surgical), while medium for OSC-IVM was prepared with all supplements (see Table below).

Table 1: Cell Culture Media Conditions

	EXPERIMENT 1: OSC activity		**EXPERIMENT 2: OSC clinical relevance**	
	Experimental Group	*Control Group*	*Experimental Group*	*Control Group*

IVM medium (Medicult, Cooper Surgical)	X	X	X	X
75 mIU/ml of recombinant FSH (Millipore, F4021)	X	X	X	X
100 mIU/ml of recombinant hCG (Sigma, CG10)	X	X	X	X
500 ng/ml Androstenedione (Sigma, A-075I)	X	X	X	
1.0 µg/ml Doxycycline (StemCell Tech., 100-1047)	X	X	X	
100,000 OSCs per 100 µl droplet	X		X	

Subject description (Experiment 1): We collected 132 oocytes from 25 subjects (average age of 25) who underwent abbreviated gonadotropin stimulation, with 49 utilized in OSC-IVM co-culture and 83 utilized in control culture. Co-culture in the Experimental and Control Conditions was performed in parallel when possible, and COCs were distributed equitably when performed in parallel, resulting in 30 unique oocyte cultures total. Equitable distribution means that COCs with distinctly large cumulus masses, small cumulus masses, or expanded cumulus masses were distributed as equally as possible between the two conditions. Other than the selective distribution of the distinct COC sizes, the COCs were distributed as randomly as possible between one or two conditions. Due to the low number of oocytes retrieved per subject in this comparison, it was often not possible to distribute oocytes equally between conditions simultaneously. COCs were subjected to these *in vitro* maturation conditions at 37ºC for a total of 24-28 hours in a tri-gas incubator with CO_2 adjusted so that the pH of the bicarbonate-buffered medium was 7.2-7.3 and with the O_2 level maintained at 5%.

Subject description (Experiment 2): For the IVM outcome endpoint, 21 subjects were recruited for the comparison. We collected 143 COCs included in the comparison, allocating 70 utilized in the commercial IVM control and 73 utilized in the OSC-IVM condition. Co–culture in the Experimental and Control Conditions was performed in parallel for all subjects. COCs were distributed equitably between the two conditions, as described above. COCs were subjected to these *in vitro* maturation conditions at 37ºC for a total of 28 hours in a tri-gas incubator with CO_2 adjusted so that the pH of the bicarbonate-buffered medium was 7.2-7.3 and with the O_2 level maintained at 5%. *In vitro* maturation with subsequent embryo formation was performed to assess developmental competence of the oocytes treated in the OSC-co-culture system in comparison to oocytes treated with commercially available IVM medium. For embryo formation, a small cohort of oocyte donors were recruited and donor sperm was utilized for fertilization. For the embryo outcomes endpoint, seven additional subjects were recruited for the comparison. We collected 49 COCs included in the comparison, allocating 23 utilized in Commercial IVM control control and 26 utilized in the OSC-IVM condition. Co–culture in the Experimental and Control Conditions was performed in parallel. COCs were distributed equitably between the two conditions, as described above. COCs were subjected to these *in vitro* maturation conditions at 37ºC for a total of 28 hours in a tri-gas incubator with CO_2 adjusted so that the pH of the bicarbonate-buffered medium was 7.2-7.3 and with the O_2 level maintained at 5%. Embryo formation proceeded in parallel, with groups kept separate, with culture proceeding no longer than day 7 post-IVM.

Assessment of oocyte *in vitro* maturation

Following the end of the 24 to 28 hour *in vitro* maturation period, COCs were subjected to hyaluronidase treatment to remove surrounding cumulus and corona cells. After hyaluronidase treatment, cumulus cells and OSCs were banked for future analysis and oocytes were assessed for maturation state according to the following criteria:

GV - presence of a germinal vesicle, typically containing a single nucleolus within the oocyte.
MI - absence of a germinal vesicle within the oocyte and absence of a polar body in the perivitelline space between the oocyte and the zona pellucida.
MII - absence of a germinal vesicle within the oocyte and presence of a polar body in the perivitelline space between the oocyte and the zona pellucida.

Oocyte morphology scoring

Following IVM, oocytes were harvested from culture dishes and stripped of cumulus cells and OSCs, assessed for maturation, then individually imaged using digital photomicrography. After imaging, oocytes were flash frozen in 0.2ml tubes prefilled with 5µl DPBS. The images were later scored according to the Total Oocyte Score (TOS) grading system.[42] Oocytes were scored by a single trained embryologist and given a score of -1, 0, 1 for each of the following criteria: morphology, cytoplasmic granularity, perivitelline space (PVS), zona pellucida (ZP) size, polar body (PB) size, and oocyte diameter. ZP and oocyte diameter were measured using ECHO Revolve Microscope software and image analysis software Fiji (2.9.0/1.53t). The sum of all categories was taken to give each oocyte a total oocyte score, ranging from -6 to +6 with higher scores indicating better morphological quality.

Oocyte endpoints following morphological scoring

For oocytes used only for evaluation of oocyte maturation, oocytes were individually placed in 0.2ml tubes containing 5µl Dulbecco's Phosphate Buffered Saline (DPBS), snap frozen in liquid nitrogen and stored at -80ºC for subsequent molecular analysis.

For oocytes used to create embryos, matured oocytes were immediately utilized for intracytoplasmic sperm injection (ICSI) and subsequent embryo formation to the blastocyst stage was evaluated. No oocytes from this study were utilized or banked for transfer, implantation, or reproductive purpose.

***In vitro* fertilization and embryo culture**

A cohort of seven subjects was utilized for *in vitro* maturation and subsequent embryo formation. The COCs from these subjects were subjected to the conditions used in Experiment 2 (treatment with OSC co-culture with all adjuvants versus commercially available IVM treatment as the control). All COCs were cultured for 28 hours then denuded, assessed for MII formation and micrographed as a group. Individual oocytes in each condition were injected with sperm via intracytoplasmic sperm injection (ICSI) on day 1 post-retrieval.[43] After ICSI, the oocytes were cultured in a medium designed for embryo culture (Global Total, Cooper Surgical, Bedminster, NJ) at 37ºC in a tri-gas incubator with CO_2 adjusted so that the pH of the bicarbonate-buffered medium was 7.2-7.3 and with the O_2 level maintained at 5%. 12 to 16 hours post-ICSI, fertilization was assessed and zygotes were micrographed, and oocytes with two pronuclei were cultured until day 3. Cleaved embryos were micrographed, underwent laser-assisted zona perforation and were allowed to develop until the blastocyst stage.[44,45] Blastocysts were assessed on day 5, 6, and/or 7, micrographed, scored according to the Gardner scale, and afterward, underwent trophectoderm biopsy for preimplantation genetic testing for aneuploidy (PGT-A) and cryopreservation if deemed usable quality (i.e. greater than or equal to a 3CC rating).[46]

Trophectoderm biopsies were transferred to 0.2ml PCR tubes and sent to an external research laboratory (Juno Genetics, Basking Ridge, NJ) for comprehensive chromosomal analysis using a single nucleotide polymorphism (SNP) based next generation sequencing (NGS) of all 46 chromosomes (pre-implantation genetic testing for aneuploidy, PGT-A).[47,48]

Data analysis and statistics

Oocyte maturation outcome data was analyzed using Python statistical packages pandas (1.5.0), scipy (1.7.3), and statsmodels (0.13.2). Maturation percentages and embryo formation outcomes by donor group were analyzed by *t-test* as functions of the IVM environment (OSC-IVM or Media control). *t-test* statistics were computed comparing cell line incubation outcomes *versus* media control, then used to calculate *p*-values, using Welch's correction for unequal variance. Bar graphs depict mean values for each population and error bars represent standard error of the mean (SEM). Number of independent oocytes for the experiment, and paired versus unpaired *t-test* parameters are indicated in the figure and Materials and Methods.

Results

hiPSC-derived OSCs effectively promote human oocyte maturation in co-culture system

In order to obtain immature COCs for IVM, we utilized similar protocols to previous studies for IVM, truncated IVF or hCG primed-IVM, namely 3-4 days of abbreviated gonadotropin stimulation and most often an hCG trigger.[19,49,50] This abbreviated stimulation program, particularly when hCG was included, yielded a mixed cohort of oocytes that were mostly immature (GV and MI), but expanded cumulus COCs were obtained as well, which may have contained MII oocytes. Oocyte donor demographics and treatment regimens are shown in Table 2 for each experimental group. Overall, the results demonstrate we were able to retrieve oocytes from non-polycystic ovarian syndrome (non-PCO/PCOS) donors, albeit at a lower yield than traditional controlled ovarian hyperstimulation cycles. In Experiment 1, oocytes from each donor were allocated to either the control IVM or OSC-IVM arm or both when possible. For Experiment 2, the control and OSC-IVM arms for both endpoints contained identical donor groups as oocytes were split equally between culture conditions for each donor. A schematic of the OSC-IVM condition is shown in Figure 1A, with a representative image of the OSC co-culture seen in Figure 1B.

Table 2: Donor Demographic and Stimulation Characteristics

	EXPERIMENT 1: OSC activity		**EXPERIMENT 2: OSC clinical relevance**	
	Experimental Group	*Control Group*	*IVM only*	*Embryo Formation*
Number of Oocyte Donors included	12	18	21	7
Oocyte Donor Age (Avg ± SEM)	26 ± 0.94	24 ± 1.1	29 ± 0.63	30.4 ± 0.95
Oocyte Donor BMI (Avg ± SEM)	22 ± 1.2	22 ± 0.52	22 ± 0.58	25.2 ± 2.42

Total rFSH used for stimulation (IU)	325-600	325-600	600	5 days Clomid plus 150-300
hCG Trigger utilized (IU)	10,000	10,000	10,000	0 IU or 2,500 IU
COCs obtained (Avg ± SEM)	7.8 ± 0.98	7.7 ± 1.03	7.0 ± 0.64	7.0 ± 1.21

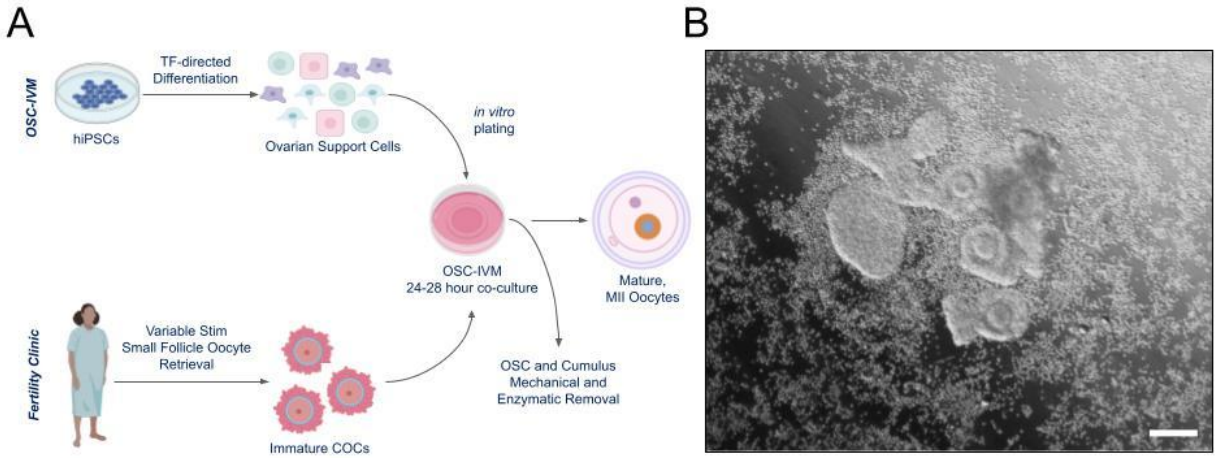

Figure 1: Ovarian support cells (OSC) co-culture system
A Schematic of the experimental co-culture IVM approach. hiPSCs were differentiated using inducible transcription factor overexpression to form ovarian support cells (OSCs). Immature human cumulus oocyte complexes (COCs) were obtained from donors in the clinic after undergoing abbreviated gonadotropin stimulation. In the lab, embryology dishes were prepared including OSCs seeding as required, and COCs were introduced for IVM co-culture. Oocyte maturation and morphological quality were assessed after 24-28 hours IVM co-culture, and samples were either banked for analysis or utilized for embryo formation.
B Representative image of co-culture setup at time of plating containing human COCs (n=5) and 100,000 OSCs. Scale bar: 100µm. COCs with expanded and unexpanded cumulus are seen with surrounding OSCs in suspension culture.

We have previously demonstrated that hiPSC-derived OSCs are predominantly composed of granulosa-like cells and ovarian stroma-like cells.[41] In response to hormonal stimulation treatment *in vitro*, the OSCs produce growth factors and steroids necessary for paracrine interaction with oocytes and cumulus cells.[41] To investigate whether hiPSC-derived OSCs are functionally capable of promoting human oocyte maturation *in vitro*, we established a co-culture system of these cells with freshly retrieved cumulus enclosed oocytes and assessed maturation rates after 24-28 hours (see Materials and Methods, Experiment 1). In this comparison, due to low numbers of retrieved oocytes per donor, we were unable to consistently split oocytes between both conditions simultaneously. Therefore each group contains oocytes from predominantly non-overlapping donor groups and pairwise comparisons are not utilized. Strikingly, we observed a statistically significant improvement (~1.5x) in maturation outcomes for oocytes that underwent IVM with OSCs (Figure 2A) compared to control. More specifically, the OSC-IVM group yielded a maturation rate of 68% ± 6.83% SEM versus 46% ± 8.51% SEM in the Media Control (Figure 2A, p=0.02592, unpaired t-test). These results support the functional activity of hiPSC-derived OSCs significantly improving oocyte maturation using this *in vitro* co-culture system.

We next examined whether hiPSC-derived OSCs would also affect the outcome of the Total Oocyte Score (TOS). Interestingly, the assessment scores (Figure 2B) were not statistically significantly different for the

two groups (unpaired *t*-test, p=0.2986), indicating that the mature MII oocytes were of equivalent morphological quality between the two IVM conditions. Altogether, these data indicate that OSC co-culture improves human oocyte maturation without a detrimental effect on morphological quality and highlights the potential for the use of hiPSC-derived OSCs as a high performing system for cumulus enclosed oocyte IVM.

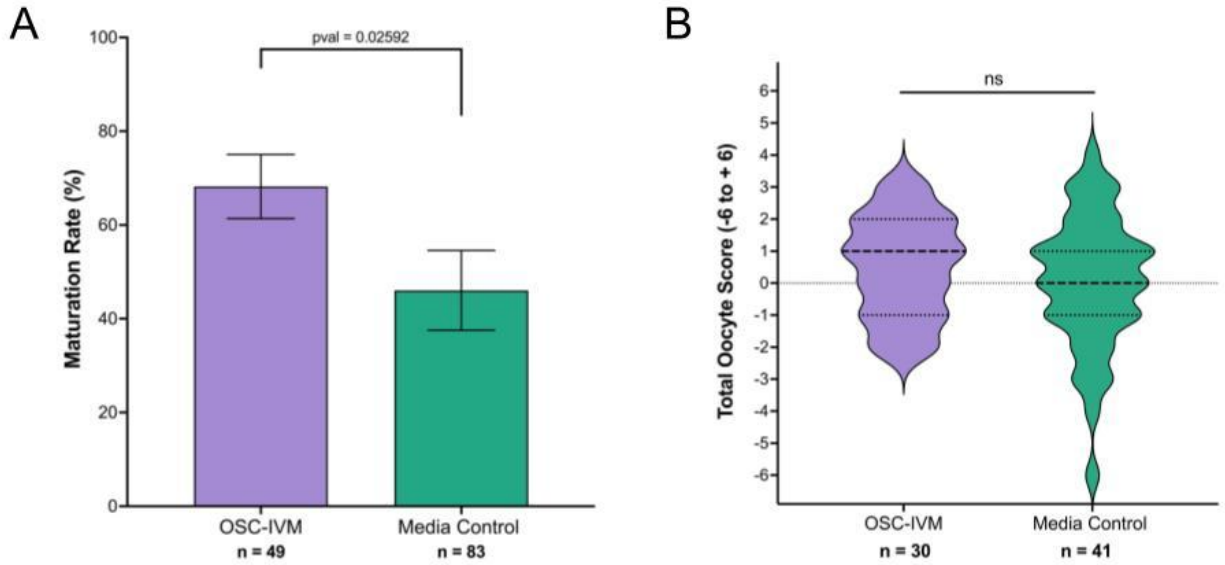

Figure 2: OSCs improve human oocyte maturation rates compared to supplemented IVM medium lacking OSCs
A Maturation rate of oocytes after 24-28 hour IVM experiments in Experiment 1, including oocytes co-cultured with OSCs, or in Media Control. *n* indicates the number of individual oocytes in each culture condition. Error bars indicate mean ± SEM. *p*-value derived from unpaired *t*-test comparing OSC-IVM to control (Media Control).
B Total Oocyte Score (TOS) generated from imaging analysis of MII oocytes after 24-28 hour IVM experiments. *n* indicates the number of individual MII oocytes analyzed. Median (dashed line) and quartiles (dotted line) are indicated. An unpaired *t*-test indicated no significant (*p*=0.2986) difference between the means. Due to low numbers of retrieved oocytes per donor, oocytes could not be consistently split between both conditions. Groups contain oocytes from predominantly non-overlapping donor cohorts thus pairwise comparisons are not utilized.

Oocyte maturation rates in OSC-IVM outperforms commercially available IVM system

To further examine the potential for using OSC-IVM as a viable system to mature human oocytes in a clinical setting, we compared our OSC co-culture system against a commercially available IVM standard. The commercially available IVM standard was utilized as described in the manufacturer's instructions for use, with no modification (Medicult IVM, Cooper Surgical). We performed a sibling oocyte study comparing the MII formation rate and oocyte morphological quality after 28 hours of *in vitro* maturation in both systems (Materials and Methods, Experiment 2). Notably, OSC-IVM yielded a statistically significant ~1.6x higher average MII formation rate with 68% ± 6.74% of mature oocytes across donors compared to 43% ± 7.90% in the control condition (Figure 3A, *p*=0.0349, paired *t*-test). Similar to our prior observations, co-culture with hiPSC-derived OSCs did not affect oocyte morphological quality as measured by TOS, indicating equivalent oocyte morphological characteristics (Figure 3B, p=0.9420, unpaired *t*-test). These results show that OSC-IVM significantly outperformed the commercially available IVM culture medium in MII formation rate with no apparent detriment to oocyte morphological quality, pointing to a beneficial application of this product for human IVM.

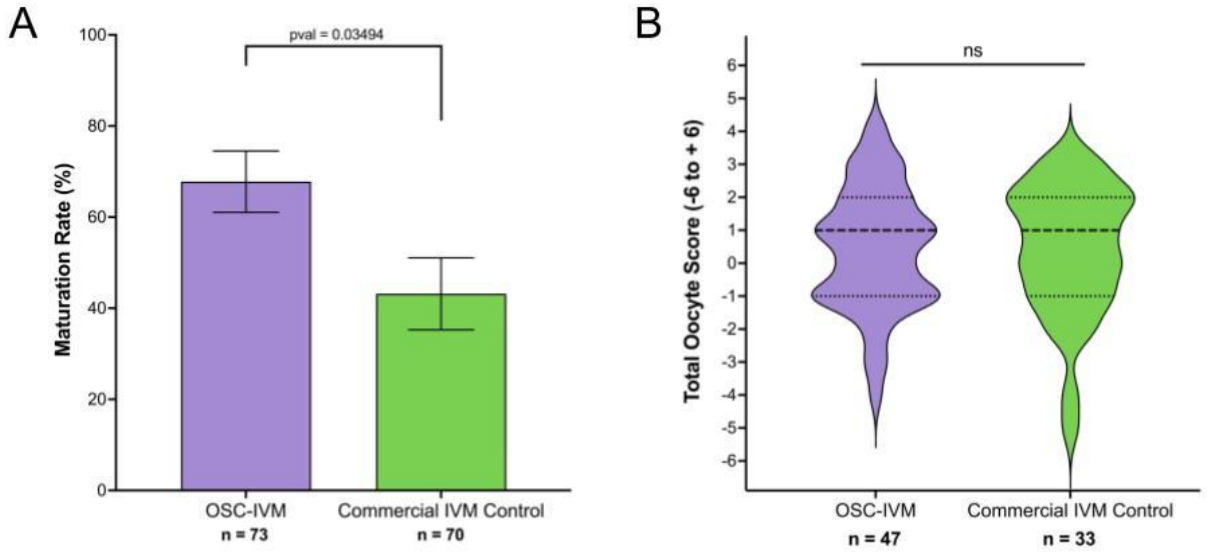

Figure 3: OSC-IVM demonstrates improved oocyte maturation compared to a commercially available IVM system
A Maturation rate of oocytes after 28 hour IVM experiments in Experiment 2, including oocyte co-culture with OSCs or in Commercial IVM Control. *n* indicates the number of individual oocytes in each culture condition. Error bars indicate mean ± SEM. p-value derived from paired *t-test* comparing Experimental OSC-IVM to control (Commercial IVM Control).
B Total Oocyte Score (TOS) derived from imaging analysis of MII oocytes after 28 hour IVM experiments. *n* indicates the number of individual MII oocytes analyzed. Median (dashed line) and quartiles (dotted line) are indicated. An unpaired *t-test* indicated no significant (p=0.9420) difference between the means. COCs from each donor were randomly and equitably distributed between control and intervention to allow for pairwise statistical comparison.

Cumulus enclosed immature oocytes from abbreviated gonadotropin stimulation matured by OSC-IVM are developmentally competent for embryo formation

We sought to investigate the developmental competency of oocytes treated in the OSC-IVM system, by assessing euploid blastocyst formation, compared to the commercially available IVM control. Utilizing a limited cohort of seven subjects who underwent abbreviated stimulation (see Materials and Methods Experiment 2, Table 1, and Table 2), we investigated whether OSC-IVM treated oocytes were capable of fertilization, cleavage, and formation of usable euploid blastocysts. We compared these embryo outcomes to those found from oocytes treated in the commercially available IVM medium. As expected, OSC-IVM yielded ~1.2x higher average MII formation rate with 61.5% ± 14% of mature oocytes across donors compared to 52.2% ± 7% in the control condition (Table 3). Matured oocytes from both conditions were immediately utilized for ICSI and followed until embryo formation (blastocyst stage). OSC-assisted MIIs demonstrate a trend towards improved fertilization (87.5% ± 6%), cleavage (100% ± 0%), blastocyst (86% ± 11%), usable-quality blastocyst (57% ± 14%), and euploid usable-quality blastocysts (57% ± 14%) formation rates compared to the commercial IVM control (75% ± 14%, 89% ± 17%, 56% ± 21%, 44% ± 18%, and 22% ± 21% respectively) (Figure 4, Table 3). Overall, in both conditions we find that in oocytes that fertilized robustly cleaved. Strikingly, PGT-A results show that of the blastocysts of usable quality generated by OSC-IVM, 100% are euploid versus 50% in the commercial IVM system. While these results are not statistically significant (p = 0.4130), likely due to the underpowered sample size for each group, these findings demonstrate that OSC-IVM generates healthy matured oocytes with high quality developmental competency. These results additionally demonstrate OSC-IVM is capable of producing healthy, euploid embryos from abbreviated stimulation cycles at a higher rate than the commercially available IVM condition, highlighting the clinical relevance of this novel system for IVM ART practice.

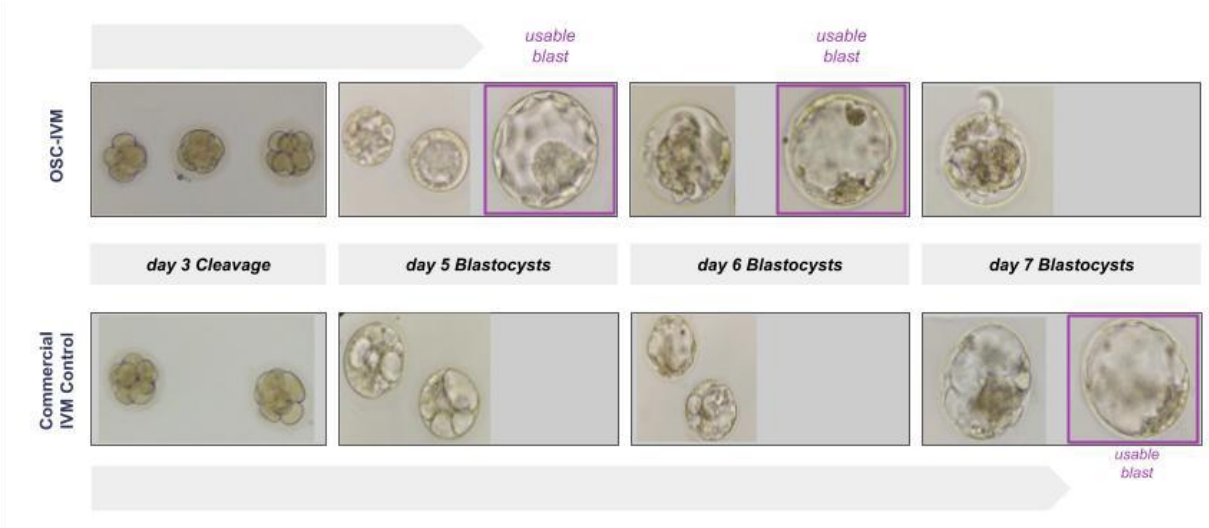

Figure 4: OSC-IVM assisted oocytes are developmentally competent for healthy embryo formation
Representative images of embryo formation in OSC-IVM versus Commercial IVM conditions at day 3 cleavage, as well as day 5, 6, and 7 of blastocyst formation. Embryos that were of suitable vitrification quality are labeled as "usable blast" and were utilized for trophectoderm biopsy and PGT-A.

Table 3: OSC-IVM oocytes are developmentally competent for euploid embryo formation

***N* = 7 Oocyte Donors**	**OSC-IVM**	**Commercial IVM Control**	***p*-value (paired *t-test)***
Number of COCs, *n*	26	23	0.1996
Maturation (MII) rate, %, (*n)*	61.5% (16)	52.2% (12)	0.4247
Fertilization rate per ICSI, %, (*n)*	87.5% (14)	75% (9)	0.3550
Cleavage rate per zygote, %, (*n)*	100% (14)	88.9% (8)	0.3739
Blastocyst rate per zygote, %, (*n)*	85.7% (12)	55.6% (5)	0.1543
Usable blastocyst rate per zygote, %, (*n)*	57.1% (8)	44.4% (4)	0.9999
Usable euploid blastocyst rate per zygote, %, (*n)*	57.1% (8)	22.2% (2)	0.4130

Discussion

It is well established that immature oocytes obtained from human ovarian follicles can mature upon extraction and *in vitro* culture.[27,51] However, the rate and extent of spontaneous oocyte maturation using these conditions are not optimized, resulting in highly unsynchronized nuclear and cytoplasmic maturation, and leaving IVM as an underperforming and underutilized technology. Current commercial IVM media have incrementally improved this process. With further optimization and development of additional culture tools, it is possible to improve the rate and extent of oocyte maturation, yielding high quality oocytes capable of healthy embryo formation. In this study, we investigate a novel IVM approach using human stem cell-derived ovarian support cells as a co-culture system for IVM of human oocytes. We demonstrated that this OSC-IVM system outperforms media without OSCs as well as a commercially available IVM culture media for MII formation. This work is the first of its kind to explore the potential of

stem cell-derived OSCs as a tool for IVM of human COCs and shows the value of this and other cell engineering approaches for application in fertility treatment. This study also demonstrated that the OSCs provided a benefit to the oocytes beyond that of the acellular supplements to the culture conditions (hormones, steroids, doxycycline). Therefore, the engineered granulosa-like cells and stromal-like cells are a beneficial addition to the *in vitro* culture environment for oocytes.

Notably, many previous IVM studies stopped at the embryo cleavage stage, whereas present ART practice increasingly relies heavily upon culture to the blastocyst stage and PGT-A as an embryo selection tool. Our study employs these more advanced selection tools. The OSC-IVM system results in MII oocytes with a high degree of developmental competence, as demonstrated by their ability to achieve fertilization, become embryos capable of cell divisions, and form blastocysts characterized with a normal euploid complement of chromosomes. In short, oocytes matured in the presence of OSCs are capable of becoming healthy euploid embryos that would likely be competent to implant and become live born babies. The ability to form healthy, euploid blastocysts is further evidence of the ability of the OSC-IVM technique to promote cytoplasmic maturation as well as nuclear maturation of oocytes, a key requirement for clinical utility of IVM systems. Further studies will explore this hypothesis. The euploidy rate in our study is of particular note, with all embryos of vitrifiable quality being euploid in the OSC-IVM condition compared to the commercial IVM control in which half of vitrified blastocysts were aneuploid. The high euploidy rate is a key indication of the potential clinical utility of OSC-IVM compared to more conventional IVM systems.

This method of IVM involving co-culture integrates readily with existing procedures employed in ART laboratories, requiring no special equipment and only minimal training for clinical embryologists. Likewise, OSC-IVM performs effectively in hCG triggered cycles with variable follicle size, which is an improvement over other IVM systems that are ill-suited for these more common stimulation styles. Our results show that OSC-IVM can be utilized across a variety of stimulation regimens, follicle extraction methodologies, and without hCG triggers, making the system highly amenable to customized abbreviated stimulation programs across the spectrum from strict IVM to truncated IVF practice. Additionally, the primarily paracrine interaction between the COCs and OSCs in this system, in which direct connections/gap junctions are not formed, affords a high degree of flexibility in co-culture format delivery and allows for a broad range of potential culture configurations.

While the number of donors and embryos generated in this study was limited, it represents an important first step in establishing stem cell derived OSCs as a co-culture platform for IVM. This study builds on previous findings that demonstrate a benefit to using primary granulosa cell co-culture for *in vitro* maturation of oocytes.[37–40] More research is needed to determine the epigenetic health of embryos generated in OSC-IVM and commercially available IVM conditions and to determine whether OSC-IVM embryos are capable of healthy implantation, development and live birth compared to commercially available IVM systems and IVF controls. Regardless of the limitations, the use of OSCs derived from hiPSCs represents a powerful approach for providing curated granulosa-like cells with characteristics more similar to a follicular rather than the luteal phase environment, as well as providing a highly controlled and robust manner for their consistent production and qualification for broad use in clinical settings.

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Author Contributions

C.C.K. designed, supervised, and coordinated the study as well as provided technical assistance in cell engineering, production, qualification, and sequencing. S.P, A.G., M.M., F.B., C.A., and M.S. performed all embryology work for the study. B.P., K.S.P., and A.N. produced and qualified OSC batches. G.R. performed all statistical analysis. A.F. coordinated study logistics. D.H.M., K.W., N.D.T., and L.G., assisted in study design, stimulation concepts, and embryology workflows. M.P.S., P.R.J.F., and P.C. assisted in OSC data interpretation S.O., D.O. and P.K. performed all oocyte donor stimulations and retrievals. C.C.K., B.P., K.S.P., G.R., and D.H.M. wrote the manuscript with significant input from all authors.

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Attestation Statement: The subjects in this trial have not concomitantly been involved in other randomized trials. Data regarding any of the subjects in the study has not been previously published unless specified. Data will be made available to the editors of the journal for review or query upon request.

Data Sharing Statement: All data needed to evaluate the conclusions in the paper are present in the paper and supplementary tables and figures. Raw and processed sequencing data will be deposited to GEO upon publication. Anonymized raw data for all findings in the paper will be provided upon request.

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