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GAMETE BIOLOGY

Effect of cumulin and super-GDF9 in standard and biphasic mouse IVM

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Abstract

Purpose In vitro maturation (IVM) is a technology that generates mature oocytes following culture of immature cumulus-oocyte complexes (COC) in vitro. IVM is characterized by minimal patient stimulation, making it attractive for certain patient groups. Recently, a biphasic IVM system, capacitation (CAPA)-IVM, has shown improved clinical outcomes relative to standard IVM; however, it remains less efficient than IVF. This study assessed whether supplementation of CAPA-IVM culture media with the novel TGFβ superfamily proteins cumulin and super-GDF9 improves subsequent mouse embryo development.

Methods Immature mouse COCs were cultured by standard IVM or biphasic IVM ± cumulin or super-GDF9.

Results Both cumulin and super-GDF9 in standard IVM significantly improved day-6 blastocyst rate (53.9% control, 73.6% cumulin, 70.4% super-GDF9; $p = 0.006$; $n = 382$–406 oocytes). Cumulin or super-GDF9 in CAPA-IVM did not alter embryo yield or blastocyst cell allocation in an unstimulated model. Moreover, cumulin did not alter these outcomes in a mild PMSG stimulation model. Cumulin in CAPA-IVM significantly increased cumulus cell expression of cumulus expansion genes (*Ptgs2*, *Ptx3*, *Adamts1*, *Gfat2*) and decreased *Lhr* expression relative to control. However, cumulin-induced mRNA expression of cumulus cell (*Ptgs2*, *Ptx3*) and oocyte genes (*Gdf9*, *Bmp15*, *Oct4*, *Stella*) in CAPA-IVM remained significantly lower than that of in vivo matured cells.

Conclusion Cumulin did not provide an additional beneficial effect in biphasic IVM in terms of blastocyst yield and cell allocation; however in standard IVM, cumulin and super-GDF9 significantly improve oocyte developmental competence.

Introduction

In vitro maturation (IVM) is an assisted reproductive technology (ART) where immature cumulus-oocyte complexes (COCs) are collected from antral follicles and matured from the prophase I to the metaphase II stage in vitro [1]. IVM is characterized by no or minimal FSH stimulation of the female prior to COC retrieval [2]. This minimal hormonal priming yields several advantages over hormone-driven conventional IVF, particularly to certain patient groups. Those to benefit most from IVM are women prone to being hypersensitive to hormonal priming, such as those with excessive antral follicle counts; women and girls who require immediate oncofertility preservation measures and are not suitable candidates for IVF; and those in countries with emerging

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economies where the cost of fertility treatment presents a greater barrier to access treatment, as IVM is a cheaper alternative to IVF (because of the reduced/eliminated gonadotropin stimulation protocol associated with it) [3, 4].

Despite its advantages, the clinical utilization of IVM has been poor, with only an estimated ~ 10,000 IVM babies born globally since 1991. The major factor contributing to its poor uptake is a lower success rate (embryo yield and live birth rate) relative to IVF [5, 6]. In IVM, small-medium sized antral follicles are targeted, which contain COCs that have not completed development, and are therefore less developmentally competent [7]. To compound this issue, IVM culture systems employed have largely provided insufficient support for the maturing COC to improve oocyte competence [8]. Recently, significant strides have been made in improving IVM culture protocols, translating into higher embryo yield and live birth rates. Arguably, the most significant advance over the past decade has been the inclusion of a pre-maturation phase prior to IVM (pre-IVM) whereby COCs are held in meiotic arrest using a cAMP- or cGMP-modulating agent, such as c-type natriuretic peptide (CNP; a meiosis inhibiting peptide), for between 2 and up to 48 h [9–16].

Animal studies demonstrate that CNP-mediated pre-IVM prolongs oocyte-cumulus cell gap-junctional communication, increases oocyte size, promotes cumulus cell proliferation and cumulus expansion, and alters mitochondrial activity, thus yielding oocytes with a greater capacity to fertilize and produce embryos [9, 10, 13]. In the mouse, CNP-mediated pre-IVM has improved embryo, implantation, and pregnancy rates [9, 10, 13]. Recently, Sanchez et al. adapted CNP-mediated pre-IVM for human oocytes to develop a system called capacitation-IVM (CAPA-IVM) which, in prospective preclinical trials, yielded increased day 3 embryo yield in PCOS patients relative to standard IVM [11, 15]. Vuong et al. [17] subsequently demonstrated that CAPA-IVM was superior to standard IVM in women with PCOS, with improved maturation and clinical pregnancy rates. A large randomised controlled trial comparing the safety and efficacy of CAPA-IVM to conventional IVF in 546 polycystic ovarian morphology (PCOM) patients found comparable outcomes in terms of pregnancy and live birth rates, although cumulative pregnancy rates were lower following CAPA-IVM than IVF [18]. This indicates that, although better than standard IVM, CAPA-IVM requires further improvement to match conventional IVF efficiency, which is needed for patients to gain the low-intervention benefits of IVM over IVF.

The oocyte plays a significant role in the orchestration of follicular development by secreting potent growth factors that regulate follicular somatic cell differentiation and function [19]. The oocyte-secreted factors, growth differentiation factor 9 (GDF9), and bone morphogenetic protein 15 (BMP15), both belonging to the TGFβ superfamily, are recognized as essential for female fertility, as genetic mutation in either one leads to altered follicle development and fertility [20, 21]. GDF9 and BMP15 act in a paracrine manner on surrounding granulosa/cumulus cells to modulate cellular functions, such as metabolism, and have been shown to improve oocyte developmental competence in standard IVM [22–24]. Because of their non-covalent dimer interaction, structural homology, similar expression patterns in the oocyte, and highly synergistic interaction, it was hypothesized that GDF9 and BMP15 form a heterodimer [25]. We engineered this GDF9:BMP15 heterodimer, called cumulin, and demonstrated its high potency on granulosa cells and that addition of cumulin to standard IVM significantly improves subsequent embryo yield in pig and mouse models [26, 27]. Human GDF9 is latent [28] and requires "activation," presumably by interacting with BMP15 [26]. Recently, we engineered a modified version of GDF9, named super-GDF9, containing BMP15 residues in the receptor binding region, which is > 1000-fold more bioactive than wildtype GDF9 and which potently activates the SMAD2/3 pathway [27]. Super-GDF9 in mouse standard IVM also improved cumulus expansion and day 5 blastocyst yield [27].

Since initial studies demonstrate that cumulin and super-GDF9 have the potential to improve standard IVM treatment, we hypothesized that cumulin and super-GDF9 supplementation would improve CAPA-IVM outcomes.

Materials and methods

To explore the effects of oocyte-secreted factors (OSFs) (cumulin and super-GDF9) on oocyte developmental competence during IVM, this study consists of three separate experiments (detailed below).

Preparation of OSFs

Cumulin and super-GDF9 were produced and purified as described previously [26, 27]. Briefly, cumulin was prepared by co-expressing wild-type human GDF9 and N-terminally His8 tagged human BMP15 in puromycin selected HEK293T cells, which were grown to confluence in serum containing growth medium, before transfer to serum-free production medium (DMEM/F12 GlutaMAX, 0.1 mg/ml BSA (Sigma), 25 IU/ml Fragmin (Pfizer), 100 units/ml penicillin/100 ug/ml streptomycin (Sigma)). After 48 h of incubation, the conditioned medium containing cumulin was collected and stored at − 20 °C. The poly-His tagged protein complexes were purified from the conditioned medium via IMAC chromatography using nickel-nitrilotriacetic acid-agarose (Invitrogen) under non-denaturing conditions, utilizing an elution buffer of PBS/500 mM imidazole.

Such purified protein samples were stored in LoBind tubes (Eppendorf) at − 80 °C.

For production of super-GDF9 [27], HEK293T cells were seeded in DMEM containing 10% FCS into 15 cm culture plates (10×10^6 cells/plate). After overnight incubation, cells were transfected with plasmid DNA (60 µg/plate) using polyethylenimine-MAX (Polysciences, Warrington, PA) in OPTI-MEM medium (Life Technologies, Carlsbad, CA), before replacement with production medium [DMEM:F-12 medium containing GlutaMAX™ (Life Technologies), 0.02% BSA, 0.005% heparin (Sigma-Aldrich)] and incubating a further 72 h before collection. Conditioned medium was concentrated and resuspended to a final volume of 5 mL in phosphate buffer [50 mM phosphate buffer, 300 mM NaCl, pH 7.4]. Super-GDF9 was then purified from the concentrated solution via cobalt-based IMAC (Thermo Fisher Scientific, Waltham, MA), followed by dialysis against phosphate buffer using a Slide-A-Lyzer® MINI Dialysis Device (Thermo Fisher Scientific).

Experiment I: testing OSFs in standard IVM with COCs collected from stimulated mice

COC collection 28-day-old C57BL/6 J female mice were stimulated with 5 IU PMSG (Folligon, Australia) for 46 h. COCs were collected, by puncturing antral follicles, in HEPES-buffered α-MEM (Gibco, Grand Island, NY, USA) supplemented with 3 mg/mL BSA (fatty acid-free bovine serum albumin; MP Biomedicals, Auckland, New Zealand), 50 µg/mL gentamycin (Sigma-Aldrich, USA), and 100 µM IBMX (3-isobutyl-1-methylxanthine; Sigma-Aldrich).

IVM IVM culture medium was prepared by supplementing bicarbonate buffered α-MEM (Gibco) with 3 mg/ml BSA (CellMaxx, MP Biomedical, New Zealand), 1 mg/ml fetuin (Sigma-Aldrich), 50 ng/ml mouse recombinant amphiregulin (mAREG; R&D Systems, MN, USA), and 50 ng/ml mouse recombinant epiregulin (mEREG; R&D systems). Both OSFs (cumulin and super-GDF9) were tested separately by IVM media supplementation at 50 ng/ml. These doses were based on a previous study which showed that 50 ng/mL was superior in inducing cumulus cell expression of genes required for cumulus expansion [27]. Groups of COCs (up to 50) were cultured in 500 µL pre-equilibrated culture media in 4-well culture dishes (NUNC, Thermo Scientific, Roskilde, Denmark) at 37 °C, 5% CO_2 in humidified air for 17 h.

IVF and embryo culture Sperm was expelled from epididymal cauda of adult CBB6F1 mice into 900 µL pre-equilibrated fertilization medium (Research Fert, IVF Vet Solutions, Adelaide, Australia) supplemented with 4 mg/ml BSA, and capacitated for 1 h at 37 °C, 5% CO_2, 5% O_2, and N_2 balance. COCs were transferred to 90 µL drops of pre-equilibrated fertilization media (supplemented with 4 mg/mL BSA) with up to 20 COCs per droplet. Capacitated sperm (10 µL) was added to the drops containing COCs, and gametes were co-incubated for 4 h at 37 °C, 5% CO_2, 5% O_2, and N_2 balance. Presumptive zygotes were then denuded and washed three times in wash medium (Research Wash, IVF Vet Solutions) supplemented with 4 mg/ml BSA and transferred in groups of 10 into 20 µL drops of cleavage medium (Research Cleave, IVF Vet Solutions) supplemented with 4 mg/ml BSA under paraffin oil (Merck, Germany). Presumptive zygotes were incubated at 37 °C, 5% CO_2, 5% O_2 and N_2 balance. On day 2 (D2) of embryo culture, cleavage rate was assessed. Embryo development was monitored on D4, D5, and D6 when morula, blastocyst, and hatching blastocyst rates were assessed, respectively. A total of 321–406 COCs were assessed per treatment over 8 biological replicate experiments.

Experiment II: testing OSFs in CAPA-IVM with COCs collected from minimally stimulated mice

COC collection 28-day-old C57BL/6 J female mice were minimally stimulated with 5 IU PMSG for 23 h. COC collection was performed as described in Experiment I.

CAPA-IVM Pre-IVM culture medium was prepared by supplementing bicarbonate buffered α-MEM with 3 mg/mL BSA, 50 µg/mL gentamycin, 1 mg/mL fetuin, 2.5 mIU/mL recombinant human FSH (FSH; Puregon, Organon, Oss, the Netherlands), 5 ng/mL insulin (Sigma-Aldrich), 10 nM estradiol (E2; Sigma-Aldrich), and 25 nM CNP (Tocris Bioscience, Abingdon, UK). IVM culture medium was prepared by supplementing bicarbonate buffered α-MEM with 3 mg/mL BSA, 50 µg/mL gentamycin, 1 mg/mL fetuin, 2.5 mIU/mL FSH, 50 ng/mL mAREG, and 50 ng/mL mEREG. Cumulin was tested in a two-by-two factorial experimental design where it was added (20 ng/mL) to either one or both of the phases of CAPA-IVM. Groups of COCs (up to 50) were cultured in 500 µL pre-equilibrated culture medium in 4-well culture dishes (NUNC) at 37 °C, 5% CO_2 in humidified air. The pre-IVM duration was 24 h, and IVM duration was 18 h.

IVF and embryo culture IVF, embryo culture, and assessment of embryo development were performed as described in Experiment I. A total of 387–424 COCs were assessed per treatment over 6 biological replicate experiments.

Embryo differential staining Differential staining was performed as described previously [9, 29], on D6 embryos to assess blastocyst cell allocation (inner cell mass (ICM) and trophectoderm (TE)) and to determine total blastomere

numbers. Briefly, blastocysts were incubated overnight with 25 µg/ml Hoechst 33,342 (in absolute ethanol) at 4 °C following a short exposure (12–15 s) to 0.5% Triton X-100. Samples were mounted with glycerol on a siliconized slide and imaged using an Olympus BX51 fluorescence microscope (Olympus Corporation, Tokyo, Japan). A total of 84–112 D6 blastocysts were analyzed per treatment over 6 biological replicates.

Experiment III: testing OSFs in CAPA-IVM with COCs collected from unstimulated mice

COC collection Ovaries were harvested from 19- to 21-day-old prepubertal F1 C57BL/6 J×CBA/ca mice without hormonal stimulation, and COCs from small antral follicles were collected by puncturing with insulin needles, into Leibovitz L15 (Sigma-Aldrich, Belgium) medium supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 µg/mL streptomycin (all from Thermo Fisher Scientific, Belgium), and 200 µM IBMX (Sigma-Aldrich). In the case where in vivo matured COCs were used as a control, mice were superovulated by 48 h administration of PMSG followed by 14 h of hCG, and COCs were collected from the oviducts.

CAPA-IVM Basal culture media was prepared with α-MEM (Thermo Fisher Scientific) supplemented with 2.5% FBS, 5 ng/mL insulin, 5 µg/mL apo-transferrin, 5 ng/mL sodium selenite, 10 nM E2 (all from Sigma-Aldrich), 2.5 mIU/mL FSH (Gonal-F; Merck, Belgium), and either 25 nM CNP-22 (Phoenix Europe, Germany) for pre-IVM culture or with 50 ng/mL mEREG (Bio-techne, UK) for IVM culture. Cumulin was tested by addition either to the pre-IVM phase (50 ng/ml) or to both phases of CAPA-IVM (pre-IVM 50 ng/mL and IVM 25 ng/mL). Super-GDF9 was tested by addition to both phases of CAPA-IVM (pre-IVM 50 ng/mL and IVM 25 ng/mL). The lower dose of cumulin in the IVM phase was based on previous work demonstrating that mRNA translation of GDF9 and BMP15 decreases from the GV to the MII stage [30]. As such, we adapted our approach in the unprimed mouse model to mimic this by using a higher concentration of cumulin and super-GDF in the pre-IVM phase (50 ng/mL) and a lower concentration in the IVM phase (25 ng/mL). COCs were cultured individually in round bottom ultra-low attachment 96-well plates (Corning, USA), with 75 µL pre-equilibrated medium, at 37 °C, 5% CO_2, and humidity. Duration of pre-IVM was 48 h and IVM was 18 h.

IVF and embryo culture IVF media was prepared by supplementing M16 media (Sigma-Aldrich) with 10 µL/mL non-essential amino acids (NEAA 100×; Invitrogen, Fisher Scientific, Belgium) and 3% BSA (fraction V powder; Sigma-Aldrich). For in vitro embryo culture (IVC), M16 media was supplemented with 10 µL/mL non-essential amino acids (NEAA 100x; Invitrogen) and 20 µL/mL essential amino acids (EAA 50x; Invitrogen). IVF and IVC dishes were prepared with several 30 µL droplets of the corresponding media under mineral oil (Irvine Scientific, Abbott, Belgium). Mature sperm, collected from the epididymal cauda of 2 adult males, was capacitated in 400 µL pre-equilibrated IVF media for 1 h. At the end of the IVM period, COCs were transferred to IVF media droplets (up to 15 COCs/droplet) and co-incubated with 15 µL of capacitated sperm. Following the co-incubation of gametes for 2.5 h at 37 °C in 5% CO_2, 6% O_2, and humidity, presumptive zygotes were denuded and transferred to IVC media, where they were washed three times prior to culturing in groups of a maximum of 15 at 37 °C in 5% CO_2, 6% O_2, and humidity. On D2 of embryo culture, cleavage rate was assessed. On D5, blastocyst development and hatching were evaluated. For cumulin, 119–134 COCs were assessed per treatment over 4 biological replicate experiments; and for super-GDF9, 141–157 COCs per treatment were assessed over 5 biological replicate experiments.

Embryo differential staining Day 5 blastocysts were fixed in 4% paraformaldehyde (Sigma-Aldrich). A washing and blocking solution (WBS) was prepared in PBS with 0.2% powdered milk, 2% normal goat serum (Gibco, Thermo Fisher Scientific), 0.1 M glycine (Calbiochem), 1% BSA, and 0.01% Triton-X (all from Sigma-Aldrich) and was used for all washing, blocking, and incubation procedures. Permeabilization was done with 0.1% Triton-X in WBS followed by a blocking step. Embryos were incubated with OCT3-4 primary antibody (Santa Cruz, sc-5279; diluted 1:200) for inner cell mass labelling, phalloidin (Thermo Fisher Scientific; diluted 1:40 in WBS) for f-actin labelling and Hoechst 33,334 (Thermo Fisher Scientific; diluted 1:1000 in WBS) for DNA staining. Samples were mounted in SlowFade Gold antifade reagent (Thermo Fisher Scientific) and imaged with a Zeiss LSM 800 confocal microscope. Full z-stacks were taken with optical sections of 2 µm and 40× magnification. For assessment of total number of blastomeres, Hoechst labeled nuclei were counted using the Blob Finder tool with an analysis pipeline for image processing, segmentation, and segment filter, in Arivis Vision 4D software (https://imaging.arivis.com/en/imaging-science/arivis-vision4d).

Cumulus cell and oocyte gene expression analyses

At the end of IVM, COCs were denuded mechanically with a mouth-controlled glass pipet. Cumulus cells (CCs) and oocytes were collected in pools from five oocytes, snap frozen and stored at − 80 °C until further processing. Additionally, age-matched female mice were stimulated with 2.5 IU PMSG (Folligon; Intervet, the Netherlands) for 48 h,

followed by 2.5 IU hCG (Chorulon; Intervet) for 14 h, and in vivo matured COCs were obtained from fallopian tubes, denuded using hyaluronidase, and CCs collected in pools from five oocytes, snap frozen, and stored at − 80 °C until further processing.

RNA extraction and reverse transcription was performed as previously described [31] with TRIzol reagent (Thermo Fisher Scientific) and iScript kit (Bio-Rad Laboratories), respectively. qRT-PCR was performed on a LightCycler 480 platform (Roche) in a 384-well plate format. For reaction mix and reaction program, the same protocol and settings were followed as previously described [31]. Primers used in this study (Table 1) were exon-spanning. 18S was used for normalization of the data in CCs, while luciferase (spiked) was used for oocytes. The $2^{-\Delta\Delta Ct}$ method was used to calculate the fold changes in mRNA with respect to the in vivo matured condition.

Statistical analysis

Data were analyzed using GraphPad Prism (GraphPad Software, CA, USA). Cleavage rates were calculated as a proportion of total COCs. Blastocyst rates (total and hatching) were calculated as a proportion of cleaved embryos. Cleavage and blastocyst rates were represented in percentages and arcsine transformed prior to statistical analysis. Effects of treatments were assessed through one-way ANOVA with Tukey's post hoc test (when more than 2 groups were involved) and *t* test (with two groups only). Embryo differential staining was analyzed through Kruskal–Wallis ANOVA. For gene expression analysis, ordinary one-way ANOVA with Tukey's post hoc test was used to analyze differences on the log2 transformed fold change values between the groups. Statistical tests were performed with 95% confidence intervals and $p < 0.05$ was considered significant. Unless stated otherwise, results are presented as mean ± standard error of the mean (SEM).

Results

OSFs improved subsequent embryo development in standard IVM

The addition of cumulin or super-GDF9 to standard IVM culture did not affect cleavage rates (Fig. 1A), but yielded significantly higher D5 and D6 blastocyst rates compared to the control group ($p < 0.05$; Fig. 1B and D). While the ratio of hatched blastocysts was only significantly higher with cumulin treatment on D5 ($p < 0.05$; Fig. 1C), both experimental groups had significantly higher rates of hatched blastocysts on D6 compared to the control group ($p < 0.05$; Fig. 1E). No differences were observed between cumulin and super-GDF9 treatments. The results indicate that these proteins are biologically active on mouse COCs and have comparable effects on oocyte developmental competence. These improved blastocyst rates lay the foundation to examine the use of OSFs in CAPA-IVM to improve oocyte competence.

OSFs did not alter embryo development in CAPA-IVM both in stimulated and unstimulated juvenile mice

OSFs were tested in two different CAPA-IVM models. The first model used 28-day-old mice minimally stimulated with PMSG for 23 h prior to COC collection. The effect of cumulin was explored using a 2-by-2 model, whereby 20 ng/ml of cumulin was added during either one of the phases of CAPA-IVM, or both phases, or none. No significant differences were observed in cleavage, morula,

Table 1 Sequences of primers used for RT-qPCR

Gene	RefSeq No	Forward primer	Reverse primer
18S	NR_003278.3	cggctaccacatccaaggaa	gctggaattaccgcggct
Luciferase	M15077	tcattcttcgccaaaagcactctg	agcccatatccttgtcgtatccc
Adamts1	NM_009621.5	cacatgcaagaagatgtcagg	ccctttgattccgatgtttc
Bmp15	NM_009757.5	cagtaaggcctcccagaggt	aagttgatggcggtaaacca
Egfr	NM_207655.2	gccacgccaactgtacctat	gccacacttcacatccttga
Gfat2	NM_013529.3	cgcctttgcactggttttca	ttcgtaccccgatgagcaag
Gdf9	NM_008110.2	taccgtccggctcttcagt	ttaaacagcaggtccaccatc
Lhr	NM_013582.3	gggacgacgctaatctcg	ccttggaaggtgccactgt
Oct4	NM_001252452.1	ccaatcagcttgggctagag	ttgatcttttgcccttctgg
Npm2	NM_181345.1	aatcactattgctacgctgaagg	cagtcctgagccgaaaagtt
Ptgs2	NM_011198.4	gggagtctggaacattgtgaa	gcacattgtaagtaggtggactgt
Ptx3	NM_008987.3	tggacaacgaaatagacaatgg	gatgaacagcttgtcccactc
Stella	NM_139218.1	gatgaagaggacgctttgga	cggggtttagggttagcttt
Zar1	NM_174877.3	ctcaggaccccggtgatt	ccgtacttctgctctaagaactgg

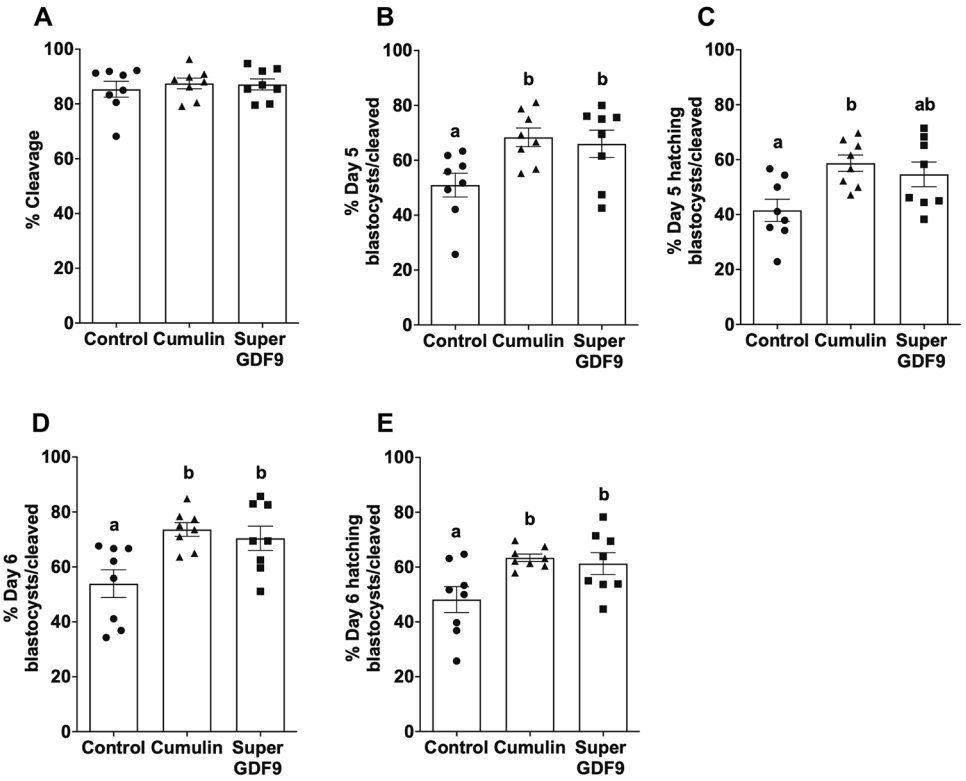

Fig. 1 Cumulin and super-GDF9 in standard IVM improved subsequent embryo development. COCs from 28-day-old mice, primed with PMSG for 46 h, were matured via standard IVM ± 50 ng/mL of cumulin or super-GDF9. Data presented as mean ± S.E.M from 382–406 oocytes per treatment across 8 biological replicates; each data point represents a biological replicate. Different letters denote a statistical significance, $p < 0.05$ one-way ANOVA of arcsine-transformed data

total blastocyst, and hatching blastocysts rates on both D5 and D6 (Fig. 2). D6 blastocysts were collected and stained to assess cell allocation. No differences were observed between the groups in the cell numbers of ICM and TE or

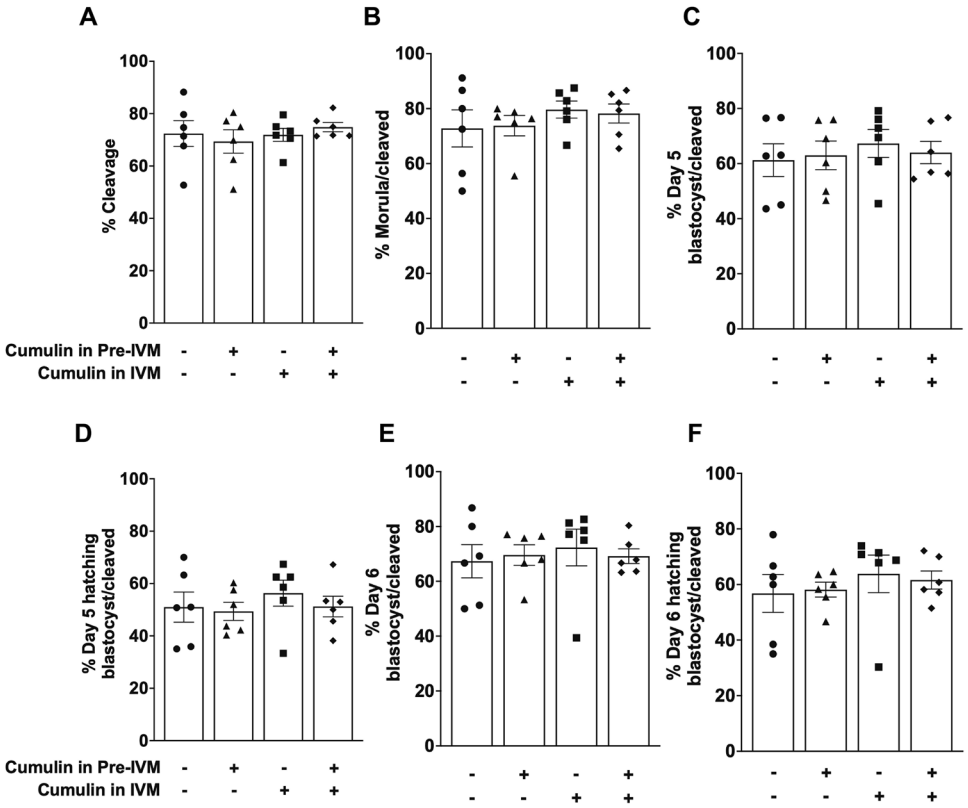

Fig. 2 Cumulin in CAPA IVM, either in the pre-IVM or IVM phase, did not alter embryo development. COCs from 28-day-old mice, primed with PMSG for 23 h, were cultured ± 20 ng/mL cumulin in the pre-IVM or IVM phase. Data presented as mean ± S.E.M from 387 to 424 oocytes per treatment across 6 biological replicates; each data point represents a biological replicate. No significant difference was found between treatments following one-way ANOVA of arcsine-transformed data

in the total number of cells (Fig. 3A–C). The ratio of ICM cells to total cells was also not different between treatments (Fig. 3D).

Next, using a model of further underdeveloped oocytes, both cumulin and super-GDF9 were tested in CAPA-IVM using younger mice (19–21 days old) and without any PMSG priming. To confirm that in vivo matured oocytes acquired from this model are competent to develop to the blastocyst stage and to ascertain IVF embryo yield, in vivo matured oocytes obtained via superovulation (48 h PMSG priming followed by 14 h of hCG) were fertilized in vitro yielding 81% cleavage and 87% D5 blastocyst rates (Fig. 4A). For CAPA-IVM, cumulin was added either during pre-IVM only or in both phases of CAPA-IVM culture. No differences were observed in cleavage, D5 total or hatching blastocyst rates, or in total blastomere numbers of D5 embryos (Fig. 4B–F). Similar results were observed with super-GDF9, since its addition to both phases of CAPA-IVM did not alter cleavage or blastocyst rates (Fig. 5A and B). Overall, these data demonstrate that cumulin and super-GDF9 do not alter subsequent embryo development during CAPA-IVM in either experimental model of oocyte competence tested.

OSFs promoted cumulus cell extracellular matrix formation

In contrast to their effects on embryo development, addition of OSFs had striking effects on the morphology of the extracellular matrix (ECM) following IVM. When immature COCs from unprimed juvenile mice were cultured with super-GDF9 during both phases of CAPA-IVM, they were demonstrably larger in size and exhibited greater mucification (Fig. 5C). Similarly, COCs cultured with cumulin during both phases of CAPA-IVM exhibited significantly different morphology compared to the controls, with greater mucification leading to highly elastic ECM (Fig. 6A).

Cumulin regulated the expression of ovulatory cascade genes

CC expression of ovulatory cascade genes in response to cumulin was examined following CAPA-IVM and was compared to post-in vivo oocyte maturation (Fig. 6). *Egfr* mRNA was similar across all treatments, indicative of the potential for equivalent receptivity to the ovulatory signal (Fig. 6B). The addition of cumulin to both pre-IVM and IVM phases of CAPA-IVM led to a significant decrease in *Lhr* and significant increases in *Gfat2*, *Ptx3*, and *Ptgs2* relative to the no-cumulin IVM control (Fig. 6C–G). This effect of cumulin was not seen if cumulin was only added to the pre-IVM phase. For these genes, the addition of cumulin to both phases mimicked the direction (if not the magnitude) of change observed in vivo (Fig. 6C–G). Together, these data demonstrate that cumulin in both phases of CAPA-IVM induces CC expression of ovulatory cascade genes which more closely resembles that of in vivo matured COCs. These results also corroborate with the notable difference in COC morphology observed (Fig. 6A).

Cumulin did not alter oocyte maturation or the expression of maternal effect genes in the MII oocyte

In the unstimulated juvenile mouse model, mean MII rate and oocyte diameter were measured following CAPA-IVM, CAPA-IVM + cumulin in pre-IVM, and CAPA-IVM + cumulin in both phases. Mean MII rates (control, 78%; cumulin in pre-IVM, 70%; cumulin in both phases, 77%) and mean oocyte diameter (Table 2) were not significantly different between treatment groups. Moreover,

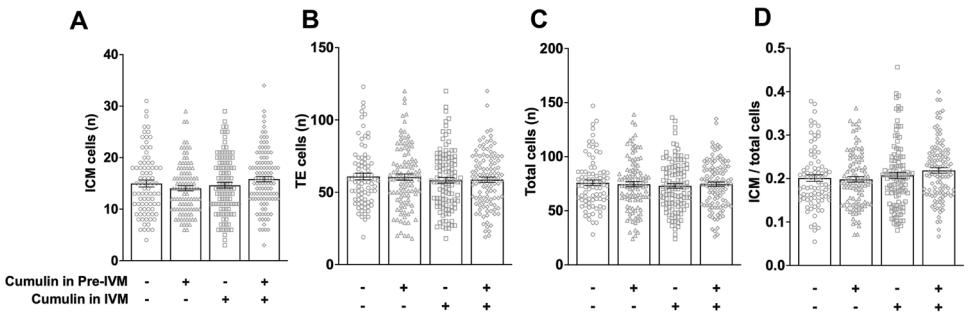

Fig. 3 Cumulin in CAPA IVM, either in the pre-IVM or IVM phase, did not alter blastocyst cell allocation. COCs from 28-day-old mice, primed with PMSG for 23 h, were cultured ± 20 ng/mL cumulin in the pre-IVM or IVM phase. Data presented as mean ± S.E.M from 84 to 112 day 6 blastocysts per treatment across 6 biological replicates; each data point represents one blastocyst. No statistical significance was found between treatments following Kruskal–Wallis ANOVA. ICM = inner cell mass; TE = trophectoderm

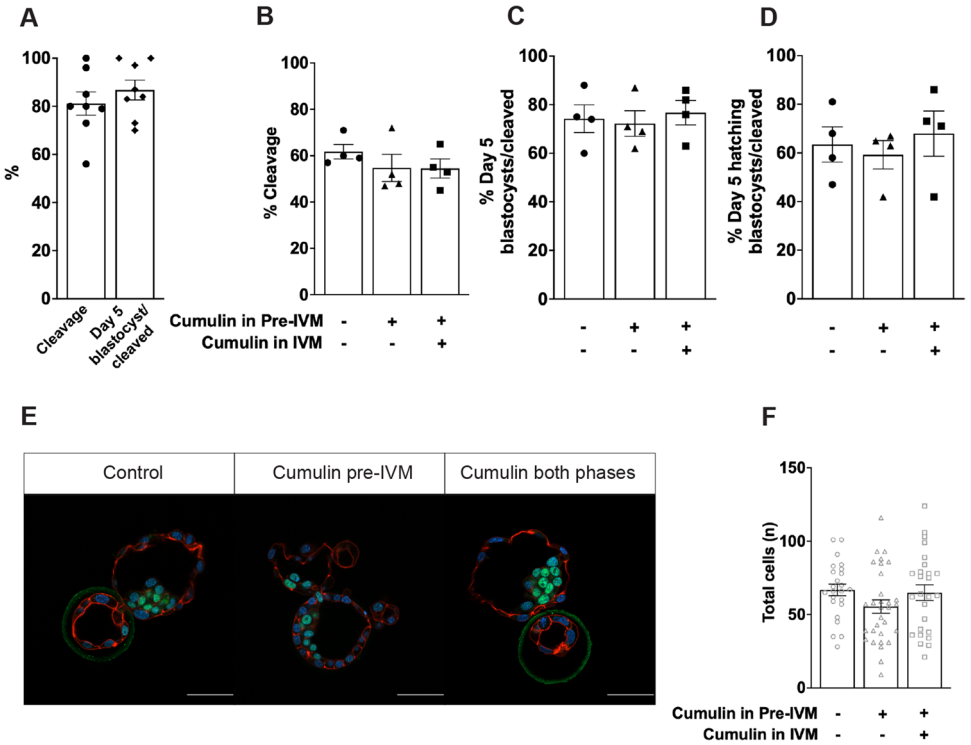

Fig. 4 Cumulin in CAPA-IVM, either only in the pre-IVM or both phases, did not alter embryo development in unprimed 19–21-day-old juvenile mice. In vivo matured COCs following superovulation were fertilised and cultured to the blastocyst stage (**A**) (data presented as mean ± S.E.M from 359 COCs across 8 biological replicates). To assess the effect of cumulin in CAPA-IVM, immature COCs from unprimed mice were cultured for 48 h ± 50 ng/ml cumulin in pre-IVM followed by 18 h ± 25 ng/ml cumulin in IVM. Cleavage rate per oocyte (**B**), day 5 blastocyst rate (**C**), and day 5 hatching blastocyst rate (**D**) were assessed. Embryo yield data presented as mean ± S.E.M from 119 to 134 oocytes per treatment across 4 biological replicates; each data point represents a biological replicate experiment. No statistical significance was found between treatments following one-way ANOVA (paired) of arcsine-transformed data. Differential staining was performed on day 5 blastocysts (**E**, **F**)**. E** Inner cell mass was labelled with OCT3-4 (green), actin filaments were stained with phalloidin (red) and nucleic acids with Hoechst 33,344 (blue). The scale bar represents 50 µm, and the original magnification is 40×, with a scan zoom of 0.7. **F** Data presented as mean ± S.E.M from 24 to 31 day 5 blastocysts per treatment across 3 biological replicates; each data point represents one blastocyst. No statistical significance was found between treatments following Kruskal–Wallis ANOVA

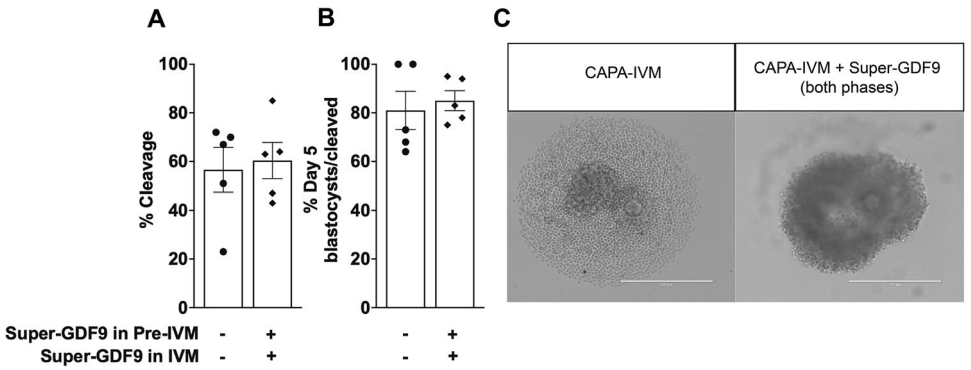

Fig. 5 The addition of super-GDF9 to both phases of CAPA-IVM did not alter embryo development but promotes ECM formation. COCs from 19- to 21-day-old unstimulated mice underwent 48 h of pre-IVM ± 50 ng/mL super-GDF9 followed by 18 h IVM ± 25 ng/ml super-GDF9. Cleavage rate per oocyte (**A**) and day 5 blastocyst rate (**B**) were assessed. Data presented as mean ± S.E.M from 141 to 157 oocytes per treatment across 5 biological replicates; each data point represents a biological replicate. No statistical significance was found between treatments following *t* test (paired) of arcsine-transformed data. COC morphology at the end of IVM was visibly different, with COCs exposed to super-GDF9 exhibiting higher mucification compared to control COCs (**C**)

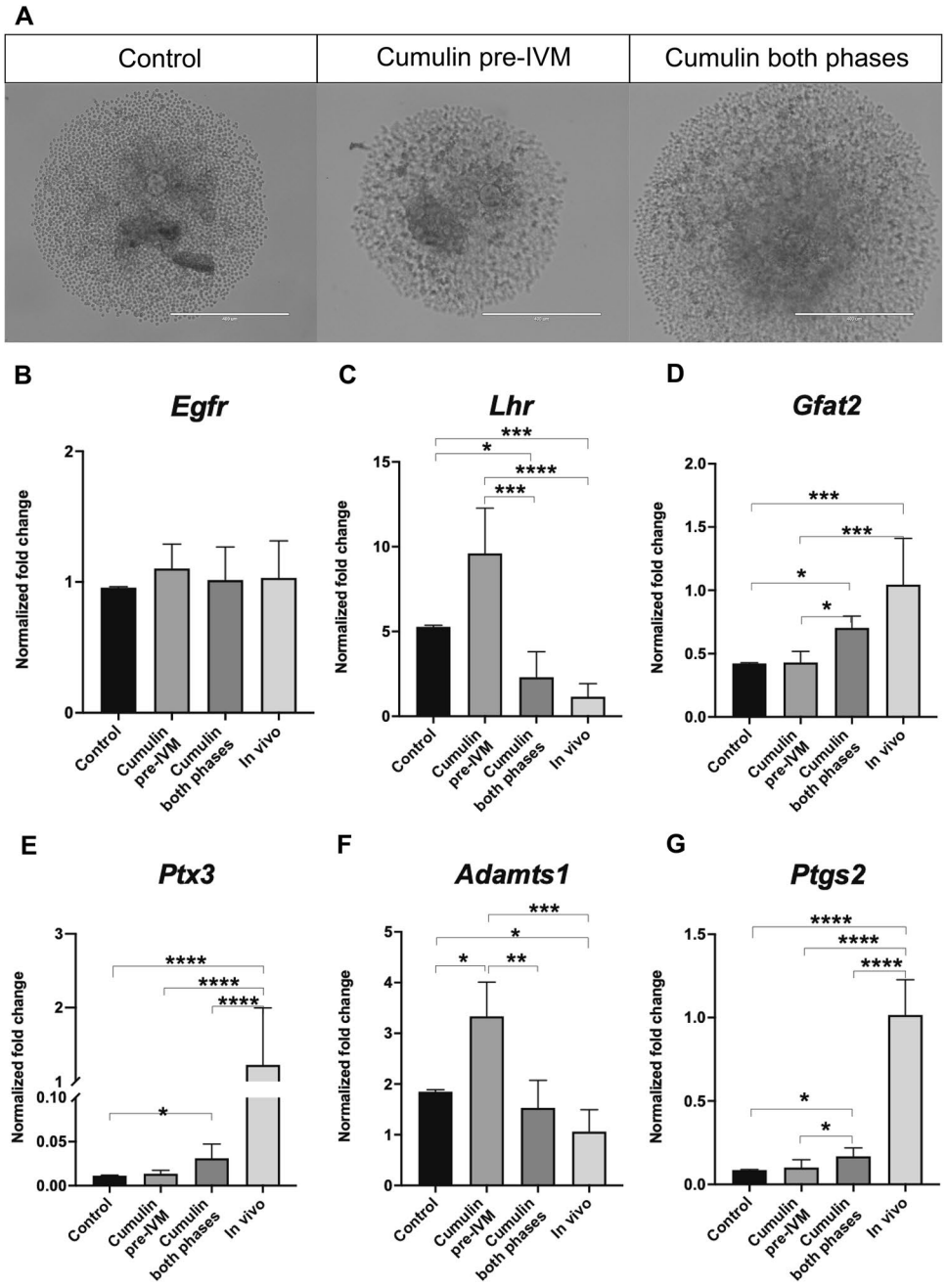

Fig. 6 Cumulin in both phases of CAPA-IVM promoted ECM formation and expression of ovulatory cascade genes in cumulus cells. COCs from 19- to 21-day-old unprimed mice were matured via CAPA-IVM ± 50 ng/ml cumulin in pre-IVM ± 25 ng/ml cumulin in IVM. COCs cultured with cumulin present in both phases exhibited more ECM mucification (**A**). At the end of IVM phase, cumulus cells from 5 COCs were pooled, and mRNA expression of genes associated with mucification were examined (**B**–**G**). Cumulus cells from age-matched in vivo matured (through superovulation) COCs were also included for comparison and data normalization was done with respect to those samples (**B**–**G**). Data presented as mean ± SD from four biological replicate experiments. Statistical difference was assessed with one-way ANOVA on the log2 transformed fold change values. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

Table 2 The diameter of oocytes from 19- to 21-day-old unstimulated mice at the end of the pre-IVM and IVM phases of CAPA-IVM

Oocyte diameter (μm)	GV, mean ± SD (*n*)	MII, mean ± SD (*n*)
Control	73.68 ± 3.16 (61)	70.76 ± 1.59 (55)
Cumulin in pre-IVM	74.16 ± 1.89 (72)	70.49 ± 1.80 (59)
Cumulin both phases		70.90 ± 1.82 (65)

the effect of cumulin on oocyte chromatin configuration was assessed using the method reported [10]; cumulin did not alter chromatin configuration as it did not significantly alter the proportion of surrounded nucleolus (SN) versus non-surrounded nucleolus (NSN) oocytes [data not shown, $n = 54$–68 GV oocytes assessed per group, collected over three independent experiments; mean SN percentage was 90 ± 4.3% for CAPA-IVM without cumulin, and 97 ± 3% for the CAPA-IVM with cumulin, $p > 0.05$].

Expression of several maternal effect genes was examined in MII oocytes obtained in the unstimulated juvenile mouse model (Fig. 7). No differences in mRNA expression were observed between CAPA-IVM oocytes matured with or without cumulin, although mRNA expression of *Gdf9*, *Bmp15*, *Stella*, and *Oct4* of CAPA-IVM oocytes

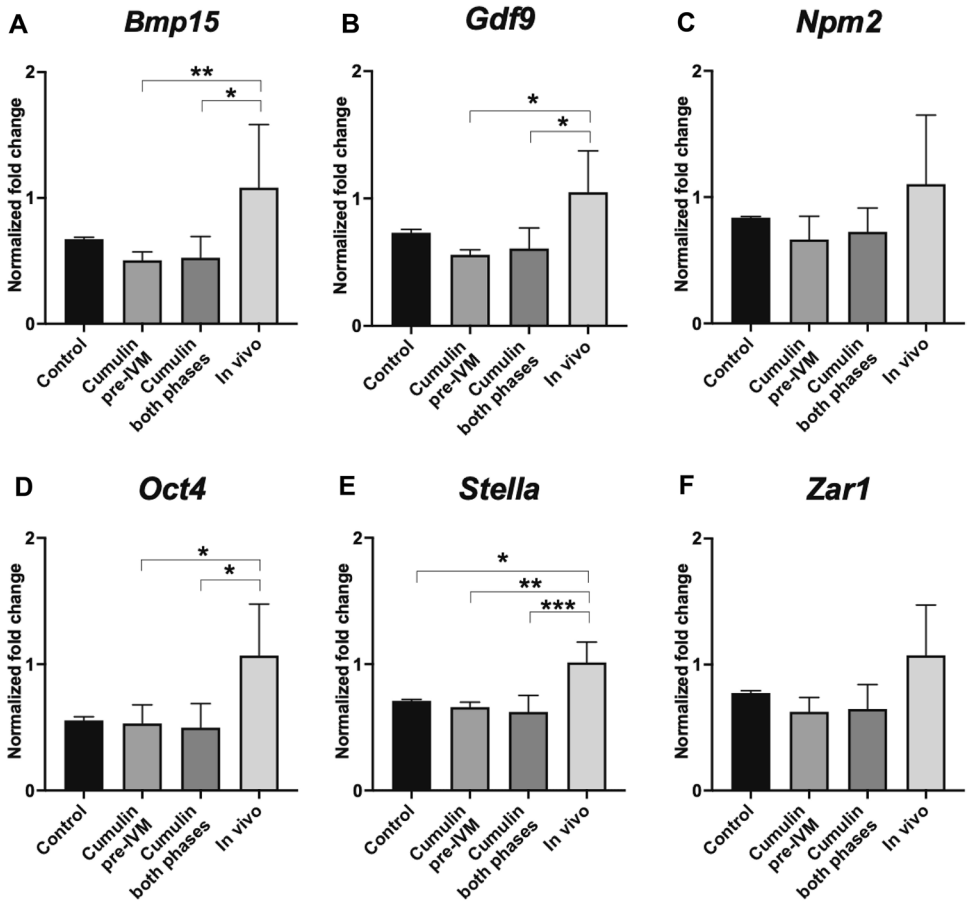

Fig. 7 Cumulin in CAPA-IVM did not alter the expression of maternal effect genes in oocytes. COCs from 19- to 21-day-old unprimed mice were matured via CAPA-IVM ± 50 ng/ml cumulin in pre-IVM ± 25 ng/ml cumulin in IVM. Cumulus cells from age-matched in vivo matured (through superovulation) COCs were also included for comparison and data normalization was done with respect to those samples. The level of mRNA transcripts for *Bmp15*, *Gdf9*, *Npm2*, *Oct4*, *Stella*, and *Zar1* (**A**–**F**) was measured. Data presented as mean ± SD from four biological replicate experiments. Statistical difference was assessed with one-way ANOVA on the log2 transformed fold change values. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

was significantly lower than that of superovulated in vivo matured oocytes (Fig. 7).

Discussion

Arguably the most significant advance in clinical IVM over the past 1–2 decades is the development of biphasic IVM, where a pre-IVM phase is introduced prior to IVM. Pre-IVM intentionally holds the oocyte meiotically arrested at prophase I in vitro for an extended period prior to IVM. The rationale for a pre-IVM phase is to promote greater synchronization between cytoplasmic and nuclear maturation by retaining and prolonging gap-junctional communication and paracrine signaling between the oocyte and cumulus cells, thus enhancing the developmental competence of the oocyte (reviewed in [32]). Initial work on the development of biphasic IVM focused on cAMP modulation in the pre-IVM phase [33–36]. However, the seminal finding that CNP (through upregulation of cGMP) is the physiological meiosis inhibiting factor in the follicle [37–39] has propelled the concept of cGMP modulation via CNP during pre-IVM. Refinement of this concept led to the development of CAPA-IVM, a novel biphasic IVM system which meiotically arrests oocytes in the pre-IVM phase using CNP prior to IVM [9–11, 13, 15]. CAPA-IVM is now in clinical use [17, 18, 40], although there is need for further improvement to the system to achieve equivalent outcomes to IVF. In an effort to address this, here we assessed the effect of cumulin and super-GDF9 in CAPA-IVM and found that cumulin and super-GDF9 did not improve CAPA-IVM embryo outcomes.

In this study, we confirmed our previous findings [26, 27] that cumulin and super-GDF9 are bioactive and their supplementation in standard IVM improves subsequent embryo yield. However, cumulin added to CAPA-IVM did not alter subsequent embryo development or blastocyst cell number or allocation. As such, cumulin and super-GDF9 appear to be beneficial in improving embryo yield during standard IVM, but not in CAPA-IVM, despite gene expression showing bioactivity on the cumulus cells. It is possible that a different dose of cumulin or super-GDF9 may endow a beneficial effect on embryo yield, although given we tried 20 and 50 ng/mL in pre-IVM, this may be unlikely. We chose to examine the effects of cumulin and super-GDF9, as opposed to GDF9 and BMP15 homodimers. This is because GDF9 and BMP15 are co-expressed by the oocyte and are more likely to operate in unison in vivo, probably in

the form of the heterodimer cumulin, rather than as isolated homodimers whose existence is questionable [26, 41–43]. In addition to activation of BMPRII and ALK6 cell-surface receptors to phosphorylate the SMAD1/5/8 transcription factors, the BMP15 subunit within cumulin activates latent GDF9 subunit, thus enabling signaling via ALK4/5 [26]. Hence, cumulin activates both SMAD2/3 and SMAD1/5/8 signaling pathways in granulosa/cumulus cells. Given the difficulty of producing a purified preparation of cumulin [44], which would be required for therapeutic use in ART media, super-GDF9 was engineered to mimic many of the actions of cumulin, as it contains BMP15 residues resulting in potent phosphorylation of the SMAD2/3 pathway [27]. Like cumulin, super-GDF9 was effective at improving oocyte quality in standard IVM, but not in CAPA-IVM.

Even though CAPA-IVM is an advanced IVM culture system, cleavage and blastocyst rates of in vivo matured oocytes were higher than those of CAPA-IVM. Furthermore, oocyte and cumulus cell gene expression levels in the current study (*Ptx3*, *Ptgs2*, *Gfat2*, *Adamts1*, *Lhr*, *Bmp15*, *Gdf9*, *Stella*, *Oct4*) were significantly different following CAPA-IVM than in in vivo matured oocytes. Hence, although it improves embryo development relative to standard IVM [9–11, 13, 15, 17], CAPA-IVM (with or without cumulin or super-GDF9) does not fully ameliorate IVM deficiencies to match in vivo oocyte maturation. A deficiency of the mouse experimental model used in the current study is the difficulty to detect improvements via the short-term endpoint used (blastocyst development), since basal embryo development rates are already high in CAPA-IVM and the assay has poor dynamic range. Future studies would benefit from moving away from short-term endpoints such as blastocyst yield and towards implantation and live birth rates. Furthermore, long-term assessments such as postnatal health of the newborns could be assessed. As such, examination of the effect of cumulin and super-GDF9 in CAPA-IVM on mouse implantation and live birth rates is still warranted as these factors may yield beneficial effects on these endpoints. Investigation into their effect on blastocyst rates in a human IVM model is still warranted since basal embryo yield rates are notably lower than mouse models.

To model mild stimulation IVM protocols (as used in human), the efficacy of cumulin and super-GDF9 was tested on oocytes from mildly stimulated (23 h PMSG-priming with 24 h pre-IVM) mice. To model zero-stimulation IVM, CAPA-IVM was performed on less developmentally competent oocytes from small antral follicles from mice which received no PMSG stimulation and a prolonged pre-IVM phase of 48 h. Full stimulation IVM (46 h PMSG-priming with standard IVM (no pre-IVM)) and in vivo matured control oocytes (46 h of PMSG + hCG) were used as controls. Although not a prerequisite for IVM, ovarian stimulation protocols are commonly applied prior to oocyte collection for clinical IVM, with a short course (2–3 days) of FSH priming being the most common approach [45], as it is well established from animal studies that exogenous FSH promotes follicular growth and oocyte quality [46–49]. However, the field lacks robust clinical trials testing the utility of FSH-priming in human IVM [50, 51]. By contrast, IVM is usually performed without prior FSH ovarian stimulation in women and girls requiring immediate fertility preservation measures who do not have the time for full ovarian stimulation, or for patients with estrogen-sensitive cancers, for whom ovarian stimulation is contraindicated. However, such oocytes are inherently less developmentally competent since competence increases with progressive follicular development, which in turn is driven by FSH (reviewed; [52]). The extent of gonadotropin priming administered prior to IVM may determine the duration of pre-IVM required to improve IVM outcomes. In fully primed mice (i.e., 48 h of PMSG), pre-IVM for 2 h yielded equal or better IVM outcomes than 24 h of pre-IVM [13], while in unprimed mice, a long pre-IVM phase of 48 h was needed [10]. Since oocyte developmental competence increases as the degree of in vivo gonadotropic priming increases [52], we speculated that the small antral follicle oocytes retrieved from unstimulated or minimally stimulated donors in the current study would be more likely to benefit from a longer, and potentially a more complex growth factor-rich, pre-IVM phase to allow further COC growth and development. For example, COCs from small antral follicles have underdeveloped EGF receptor signalling, which is enabled by OSFs [53–55], such that during IVM of such COCs, the addition of GDF9 + BMP15 enables them to respond to the EGF-like peptide amphiregulin, improving their competence [56, 57]. Despite the under-developed state of the oocytes used in the two CAPA-IVM models, neither cumulin nor super-GDF9 altered embryo development or blastocyst cell allocation in either model, including when cumulin was present in just the pre-IVM phase. Furthermore, although CAPA pre-IVM has been shown to promote an increase in mouse and human oocyte diameter [10, 15], cumulin did not alter oocyte diameter following pre-IVM or IVM, nor did it alter the proportion of mature oocytes in the zero-stimulation model.

It remains unclear why cumulin and super-GDF9 were found to be beneficial in standard IVM but not CAPA-IVM. OSFs including cumulin and super-GDF9 act on cumulus cells and presumably not the oocyte in IVM. With minimal or no gonadotropin-priming, the cumulus cells in the CAPA-IVM models might be less differentiated than those in the standard IVM model. Less differentiated cumulus cells are likely receptive to cumulin

and super-GDF9 as gene expression was altered in these cells, but perhaps only fully differentiated cumulus cells can elicit the beneficial effects of OSFs on oocyte competence. CAPA-IVM promotes oocyte growth, cumulus cell growth, and development, alters gene expression, metabolism, and prolongs cumulus-oocyte gap-junctional communication during the pre-IVM phase, which likely collectively underlies its positive effect on oocyte development [9–13, 58, 59]. But despite the progression of development of cumulus cells in the pre-IVM phase of CAPA-IVM, cumulin was nonetheless ineffective at promoting oocyte developmental competence (as assessed by blastocyst yield), whether added in the IVM phase only or in both phases. Hence, further refinement of the CAPA-IVM system is likely required, with the objective of supporting further cumulus cell development in vitro to an extent that the COC can then benefit from the addition of exogenous OSFs in CAPA-IVM. As well as the use of supplements such as cumulin and super-GDF9, improvements may also be found by further refinement of the durations of the two phases of CAPA-IVM [9, 58], to match the degree of gonadotropin priming the COC was exposed to. The duration of gonadotropin priming of the donor prior to oocyte collection, and hence the degree of oocyte developmental competence at the time of collection, is likely to influence whether oocytes require a longer pre-IVM phase to support in vitro COC capacitation [60].

Although cumulin did not increase embryo yield, it did exert some effects on cumulus cell function. Exposure to cumulin during the IVM phase shifted the gene expression profile somewhat toward that of in vivo matured COCs. Elevated expression of genes involved in cumulus matrix formation (*Ptgs2*, *Ptx3*, *Adamts1*, *Gfat2*) was seen in the presence of cumulin in the IVM phase, and, in line with this, cumulus expansion was greater. This supports our previous finding that cumulin significantly increases cumulus expansion in murine and porcine IVM [26, 27]. Cumulus expansion in response to the LH surge is a prerequisite for ovulation, and inappropriate synthesis of components of the cumulus matrix compromises fertility [61]. Oocyte-secreted factors, including GDF9 and BMP15, play an indispensable role in cumulus matrix formation (reviewed by [19]). Cumulin exposure during both phases also significantly decreased the abundance of the LH receptor to levels comparable with in vivo matured cumulus cells, demonstrating that cumulin during IVM helps cumulus cells retain their characteristic non-mural phenotype [62].

In conclusion, although cumulin and super-GDF9 did not improve embryo development in CAPA-IVM in this study, their beneficial effects in standard IVM are confirmed. This study also highlights the difficulty and complexities of developing oocyte culture technologies that are capable of enhancing both development and maturation of the oocyte in vitro. Despite the findings from this study, further efforts to exploit the use of cumulin and/or super-GDF9 in human IVM, either standard or biphasic, appear warranted given the health and economic benefits of improving human IVM.

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