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GAMETE BIOLOGY

Dysregulation of bisphosphoglycerate mutase during in vitro maturation of oocytes

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Abstract

Purpose Oxygen is vital for oocyte maturation; however, oxygen regulation within ovarian follicles is not fully understood. Hemoglobin is abundant within the in vivo matured oocyte, indicating potential function as an oxygen regulator. However, hemoglobin is significantly reduced following in vitro maturation (IVM). The molecule 2,3-bisphosphoglycerate (2,3-BPG) is essential in red blood cells, facilitating release of oxygen from hemoglobin. Towards understanding the role of 2,3-BPG in the oocyte, we characterized gene expression and protein abundance of bisphosphoglycerate mutase (Bpgm), which synthesizes 2,3-BPG, and whether this is altered under low oxygen or hemoglobin addition during IVM.

Methods Hemoglobin and Bpgm expression within in vivo matured human cumulus cells and mouse cumulus-oocyte complexes (COCs) were evaluated to determine physiological levels of Bpgm. During IVM, Bpgm gene expression and protein abundance were analyzed in the presence or absence of low oxygen (2% and 5% oxygen) or exogenous hemoglobin.

Results The expression of Bpgm was significantly lower than hemoglobin when mouse COCs were matured in vivo. Following IVM at 20% oxygen, Bpgm gene expression and protein abundance were significantly higher compared to in vivo. At 2% oxygen, Bpgm was significantly higher compared to 20% oxygen, while exogenous hemoglobin resulted in significantly lower Bpgm in the COC.

Conclusion Hemoglobin and 2,3-BPG may play a role within the maturing COC. This study shows that IVM increases Bpgm within COCs compared to in vivo. Decreasing oxygen concentration and the addition of hemoglobin altered Bpgm, albeit not to levels observed in vivo.

Keywords Oocyte maturation · Cumulus-oocyte complex · Oxygen · Hemoglobin · 2,3-Bisphosphoglycerate · Bisphosphoglycerate mutase

Introduction

To maintain an optimal environment for oocyte maturation, it is vital to understand the in vivo ovarian follicular environment and how to mimic those conditions in vitro. In vivo, the cumulus-oocyte complex (COC) is thought to be in a low oxygen environment [1]. It is located within the ovarian follicle and separated from the nearest vasculature by granulosa cells, a basement membrane, and theca cells. During folliculogenesis, the follicle increases in size, developing a fluid-filled antrum which further separates the COC from its nearest oxygen source. In humans, increasing follicle diameter is associated with a decrease in oxygen partial pressure in follicular fluid [2], which is estimated to be between 1.5 and 6.7% during development [3]. Thus, the final stages of oocyte maturation in vivo likely occur in a low oxygen environment, and in vitro maturation (IVM) of COCs at a similar oxygen level might be more physiologically relevant.

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Our understanding of oxygen regulation pathways during in vivo maturation of the COC remains limited and requires further investigation if we are to replicate this during IVM. Low oxygen levels during IVM results in different metabolic pathways being utilized by the COC, and decreased generation of damaging reactive oxygen species [4]. There are several factors that allow the in vivo matured COC to survive in a low oxygen state, notably the expression of oxygen-regulated genes [5, 6]. Significant induction of oxygen-regulated genes occurs when IVM is conducted at physiologically low oxygen (2% or 5%) [7]. This indicates that lower oxygen during IVM might be beneficial to oocyte development by mimicking the in vivo environment [8].

Interestingly, the oxygen-binding protein hemoglobin (Hb), while abundant in the in vivo matured COC, is significantly lower in vitro [9, 10]. This raises the intriguing possibility of a more sensitive regulation of oxygen supply to the COC on a biochemical scale. To add further intrigue, while upregulation of Hb expression occurs during in vivo maturation, the inverse occurs for bisphosphoglycerate mutase (Bpgm) [10]. Bpgm synthesizes 2,3-BPG (2,3-bisphosphoglycerate), the molecule that binds to Hb allosterically, allowing it to release oxygen [11]. Given that levels of Hb during IVM are low, an upregulation in Bpgm might increase levels of 2,3-BPG resulting in more oxygen being released via binding of 2,3-BPG to Hb. This mechanism is observed in human and mouse placenta facilitating increased oxygen transfer from maternal Hb to fetal Hb [12]. To date, there has been no research describing the combined role of Hb and 2,3-BPG in regulating oxygen supply during IVM.

An interplay between Hb and 2,3-BPG in regulating oxygen supply is demonstrated in other tissues. This may similarly occur in the in vivo matured COC. However, following IVM, Hb expression is dysregulated, and the levels of Bpgm are not known. Here, we characterize Bpgm during oocyte maturation by (1) comparing Hb and Bpgm gene expression in human cumulus cells and mouse COCs following in vivo maturation; (2) determining whether Bpgm is dysregulated during IVM of mouse COCs; and (3) evaluating the effect of low oxygen and exogenous Hb on Bpgm gene expression and protein abundance during IVM.

Methods

Human cumulus cells

Human cumulus cells were isolated from oocytes matured in vivo for patients undergoing in vitro fertilization (IVF; Fertility SA, Adelaide, Australia). Patients had given prior consent for their normally discarded cumulus cells to be stored and used for research purposes (Fertility SA BioResource Human Research Ethics Committee Project No. 93). The cohort comprised a total of 40 patients (age: 29–34 years) who were being treated for infertility and not donors. Patient demographics and causes for infertility are summarized in Supplementary Table 1. Ethics approval for the study was obtained from the Human Research Ethics Committee, University of Adelaide, Adelaide, Australia (approval number H-2018-205). Samples were stored at −80 °C until RNA extraction as described below.

Animals

Female (CBA × C57BL/6) first filial (F1) mice (3–4 weeks old) were obtained from the University of Adelaide Laboratory Animal Services and maintained under 12L:12D conditions with rodent chow and water provided *ad libitum*. All experiments were approved by the University of Adelaide Animal Ethics Committee (M-2016-147) and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Media

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The base medium used for all mouse ovary collection, handling, and in vitro maturation (IVM) was alpha Minimal Essential Medium (αMEM, Gibco by Life Technologies, CA, USA). The handling medium consisted of filtered HEPES-buffered αMEM medium supplemented with $NaHCO_3$, gentamicin sulfate, glucose, glutamax (Gibco by Life Technologies, CA, USA), 4 mg/ml bovine serum albumin (BSA, MP Biomedicals, AlbumiNZ, Auckland, NZ), and 1 mg/ml fetuin. Mouse IVM culture medium consisted of bicarb-buffered αMEM supplemented with gentamicin sulfate, glucose, glutamax, 3 mg/ml BSA, and 1 mg/ml fetuin. The media was filtered before adding 50 mIU/ml Puregon recombinant human follicle-stimulating hormone (rhFSH) (Organon, Oss, The Netherlands) hereafter referred to as "IVM medium." For experiments where hemoglobin was a treatment, 1 μg/ml ferrous (Hb^{2+}, Cat #H0267) or ferric (Hb^{3+}, Cat #H7379) hemoglobin was added at this step, as per published literature [10, 13]. Ferrous hemoglobin contains heme iron in the Fe^{2+} oxidation state which is capable of binding oxygen, and ferric hemoglobin contains heme iron in the Fe^{3+} oxidation state which cannot bind oxygen.

In vivo maturation of mouse COCs

Mice were injected intraperitoneally (i.p.) with 5 IU Folligon equine chorionic gonadotrophin (eCG) purchased from Pacific Vet Pty Ltd. (Braeside, VIC, Australia). At 46 h post-eCG, mice were injected with 5 IU Pregnyl human chorionic gonadotropin (hCG) from Merck (Kilsyth, VIC, Australia) (i.p.). After 16 h post-hCG, mice were culled by

cervical dislocation and ovaries dissected in the warmed handling medium. Post-ovulatory COCs were isolated by puncturing the ampulla of oviducts with a 29-gauge x ½ in. insulin syringe with needle (Terumo Australia Pty Ltd., NSW, Australia). All handling procedures were performed on microscopes fitted with warming stages calibrated to maintain the media in dishes at 37 °C.

In vitro maturation of mouse COCs at low oxygen

At 46 h post-eCG injection, mice were culled by cervical dislocation and dissected ovaries collected in the warmed handling medium for COC isolation. The COCs were isolated from ovaries by puncturing antral follicles in handling medium with insulin needles. Culture media drops were prepared at a density of 10 COCs per 500 μl of IVM culture medium under paraffin viscous oil (Merck Group, Darmstadt, Germany). Dishes were pre-equilibrated for at least 4 h in a 20% O_2, 6% CO_2, and balance of N_2 humidified 37 °C incubator. The COCs were matured for 16 h in 2, 5, or 20% oxygen (6% CO_2 and balance of N_2). To maintain 2% oxygen during IVM, a separate gas cylinder containing 2% oxygen, 6% CO_2, and balance of N_2 was gassed into a humidified modular incubation chamber containing COCs in culture dishes (Billups-Rothenburg, Del Mar, CA, USA) and placed into a 37 °C incubator [14]. For time-course experiments, COCs were cultured in 2% oxygen, 6% CO_2 and balance of N_2 gassed MINC mini incubators (Supplementary Fig. 2). To maintain 5 and 20% oxygen levels, two separate incubators set at the respective oxygen levels were used.

Gene expression analysis

Mouse COCs (40 per replicate) were snap frozen in liquid nitrogen and stored at −80 °C until RNA isolation. Extraction of RNA from snap frozen COCs and human cumulus cells was carried out using Qiagen RNAeasy Mini Kit (Cat #74104, Qiagen, Chadstone Centre, VIC, Australia), as per manufacturer's instructions. Quantification of RNA was performed using a Nanodrop 2000 Spectrophotometer (Thermo Fisher). Eluted RNA was reverse-transcribed to cDNA using random hexamer primers and SuperScript III reverse transcriptase (Invitrogen, Life Technologies, CA, USA) as per manufacturer's instructions for First-Strand cDNA Synthesis. The cDNA was either stored at −20 °C at this step or proceeded to gene expression analysis.

Gene expression analysis of COCs obtained from IVM at different oxygen concentrations was evaluated using SYBR Green Real-Time qPCR. For each sample, 1 μl diluted cDNA (1:2 with RNase-free H_2O as per prior literature [10]), 0.1 μl forward and reverse primers (25 μM), 5 μl SYBR Green Master Mix (Applied Biosystems, CA, USA), and RNAse-free water were added to a final volume of 10 μl, then loaded into striptubes (Qiagen, Hilden, Germany). Reactions were performed in triplicate. Expressions of hemoglobin alpha protein (*Hba-a1*), hemoglobin beta protein (*Hbb*), bisphosphoglycerate mutase (*Bpgm*), glucose transporter-1 (solute carrier family 2 (facilitated glucose transporter), member 1) (*Slc2a1*), and N-Myc downstream-regulated gene 1 (*Ndrg1*) were analyzed. Primer sequences, found in Table 1, were designed using mRNA sequences from the National Center for Biotechnology Information PubMed database using the Primer Express software (PE; Applied Biosystems, Foster City, CA) and synthesized by Integrated DNA Technologies (Singapore, Singapore). Conditions for PCR followed a two-step with melt protocol: 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 45 s, and ending with a melt step at 72–90 °C on a Rotor-Gene 6000 (Corbett Life Science, NSW, Australia). Single-product amplification was confirmed by analysis of melt dissociation curves. Gene expression was calculated relative to 60s ribosomal protein L19 (*Rpl19*) expression using the $2^{-(\Delta CT)}$ method [15]. Housekeeping gene stability was analyzed using the Bestkeeper housekeeper analysis software [16]. A negative control with no cDNA template was included and showed no evidence of amplification.

TaqMan Real-Time qPCR (Applied Biosystems, CA, USA) was used to evaluate gene expression of human cumulus cell samples. Each sample consisted of 5 μl of TaqMan Gene Expression Master Mix, 0.5 μl of TaqMan Gene Expression Assay (Thermo Fisher Scientific, MA USA) (see Table 2 for all TaqMan gene expression assay IDs used), 2.5 μl H_2O, and 2 μl cDNA (2.5 ng/μl, as per manufacturer recommendations and prior literature [17]). The PCR was performed on a QuantStudio 12K Flex (Applied Biosystems, CA, USA) analyzed using comparative CT with housekeeper glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The thermal profile was 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Negative controls included omission of template and cDNA samples where reverse transcriptase was omitted; these showed no evidence of amplification.

BPGM immunofluorescence

Following maturation in vivo or in vitro, COCs (20 per replicate) were fixed in 200 μl 4% paraformaldehyde diluted in phosphate-buffered saline (PBS) for 30 min, followed by washing with 200 μl 0.3 mg/ml polyvinylalcohol (PVA) in PBS (PVA/PBS). The COCs could be stored at 4 °C at this step or proceeded to blocking with 10% goat serum diluted in PVA/PBS for 30 min at room temperature. A blocking serum was also used to make up

Table 1 Primer sequences for SYBR Green qPCR analysis of gene expression in mouse COCs

Gene	Genbank accession no.	Primer sequence
Hba-a1	NM_001083955.1	Forward – AAGCCCTGGAAAGGATGTTT Reverse – GGCTCAGGAGCTTGAAGTTG
Hbb	NM_008220.4	Forward – GCTGGTTGTCTACCCTTGGA Reverse – ACGATCATATTGCCCAGGAG
Bpgm	NM_007563.4	Forward – ACCGGAGGTACAAAGTGTGC Reverse – CTCCAGCAGAATCGGAACTC
Ndrg1	BC015282	Forward – AGTACTTTGTGCAGGGCATGG Reverse – AGGGATGTGACACTGGAGCC
Slc2a1	M23384	Forward – CCAGCTGGGAATCGTCGTT Reverse – CAAGTCTGCATTGCCCATGAT
Rpl19	NM_026490	Forward – TTCCCGAGTACAGCACCTTTGAC Reverse – CACGGCTTTGGCTTCATTTTAAC

Hba-a1 hemoglobin alpha protein, *Hbb* hemoglobin beta protein, *Bpgm* bisphosphoglycerate mutase, *Slc2a1* glucose transporter-1 (solute carrier family 2 (facilitated glucose transporter), member 1), and *Ndrg1* N-Myc downstream-regulated gene 1

BPGM antibody (Cat #C-4 sc-373819, Santa Cruz Biotechnology, TX, USA) at a 1:200 dilution. A blocking peptide (Cat #sc-373819-P, Santa Cruz Biotechnology, TX, USA) was used as a negative control by pre-binding the Bpgm antibody. Incubation with the primary antibody occurred overnight at room temperature. A negative control with omission of the primary antibody was also performed. Following overnight incubation, COCs were washed three times with PVA/PBS and incubated with secondary antibody AlexaFluor488 goat anti-mouse IgG (Cat #A11001, Thermo Fisher Scientific, MA, USA) at 1:500 dilution in 10% goat serum for 2 h at room temperature in the dark. Following incubation, COCs were washed three times with PVA/PBS, mounted onto glass slides using a secure-seal spacer (Life Technologies, CA, USA) using a DAPI mounting medium, and overlaid with a coverslip. Slides were either stored at −20 °C or proceeded directly to imaging on a Fluoview FV10i confocal microscope (Olympus Life Science, Tokyo, Japan). Image processing and analyses were performed using the Fiji ImageJ software (National Institute of Health, Maryland, USA) with the plugin/macro option to remove the background and convert images to 8-bit. Macros for analyzing image fluorescence intensity, and file processing and measurements were sourced from Sutton-McDowall [18]. Images were captured at ×60

Table 2 TaqMan gene expression assay ID for gene expression analysis

Gene description	Origin	Taqman gene expression assay ID
HBA1/HBA2	Human	Hs00361191_g1
BPGM	Human	Hs00156139_m1
GAPDH	Human	Hs02786624_g1

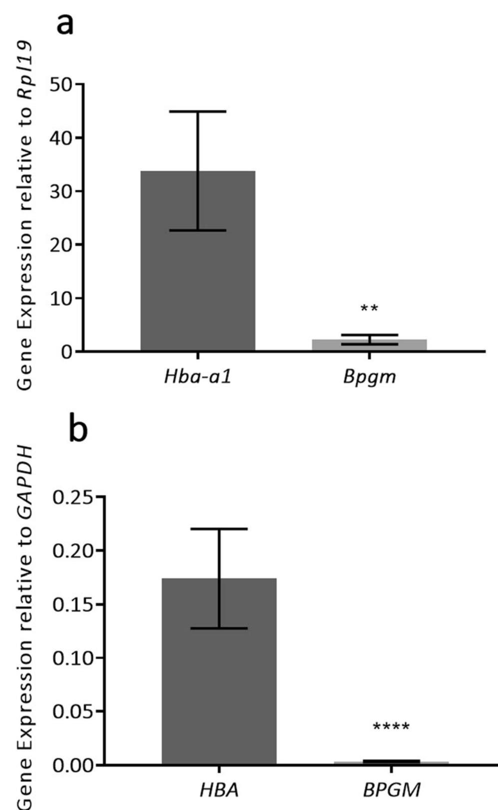

Fig. 1 Contrasting hemoglobin alpha (*Hba-a1*/*HBA*) and bisphosphoglycerate mutase (*Bpgm*/*BPGM*) gene expression in mouse cumulus-oocyte complexes (COCs) and human cumulus cells following in vivo maturation. Gene expression was measured in COCs following maturation in vivo (16 h; **a**) or human cumulus cells harvested from oocytes following in vivo maturation and follicle aspiration (**b**). The expressions of *Hba-a1* and *Bpgm* in mouse COCs are presented relative to ribosomal protein L19 (*Rpl19*) and analyzed using $2^{-\Delta CT}$ (n = 5 experimental replicates). In vivo matured human cumulus cell gene expressions of *HBA* and *BPGM* are expressed relative to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and analyzed using $2^{-\Delta CT}$ (n = 26). All data are presented as mean ± SEM. Data were log-transformed to normalize prior to statistical analysis using an unpaired Student's *t*-test, **: $P < 0.005$, ****: $P < 0.00005$

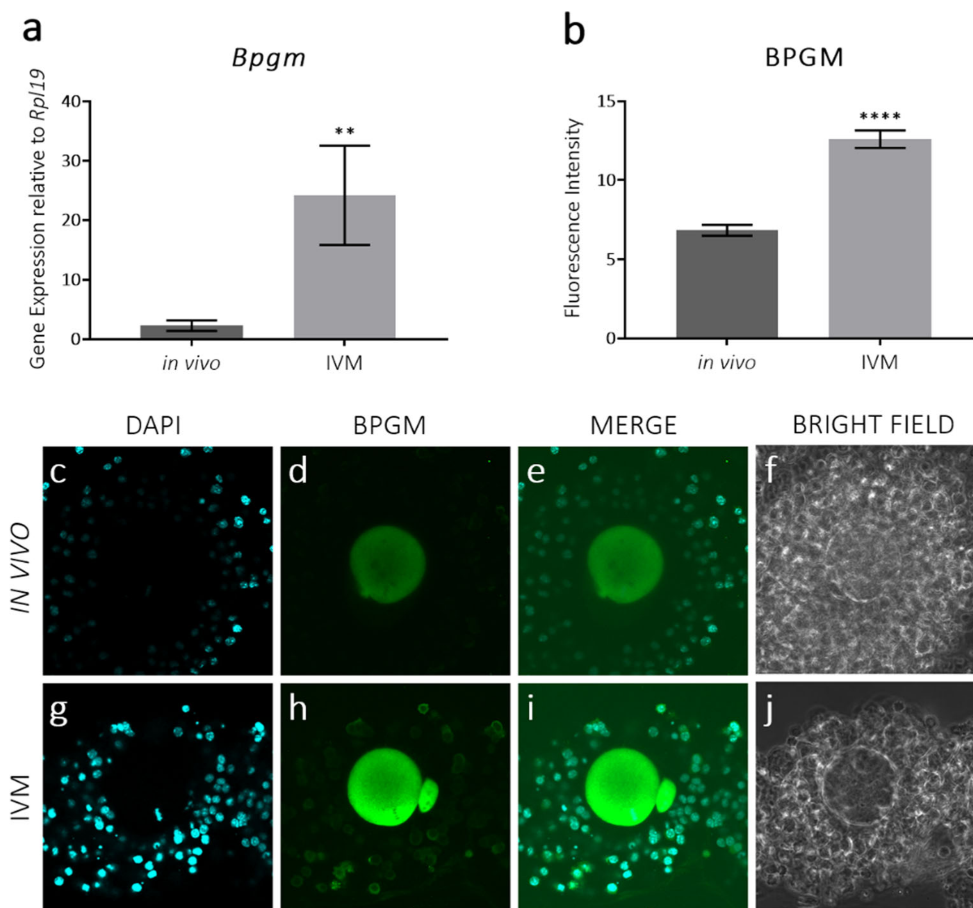

Fig. 2 Bisphosphoglycerate mutase (Bpgm) mRNA and protein are dysregulated in cumulus-oocyte complexes (COCs) following in vitro maturation (IVM). Gene expression (**a**) and protein abundance (**b**–**j**) of Bpgm were assessed in mouse COCs following in vivo maturation (16h; **a**–**f**) or IVM (16 h; **a**–**b** and **g**–**j**). The gene expression of *Bpgm* is presented relative to ribosomal protein L19 (*Rpl19*) and analyzed using $2^{-\Delta CT}$ (n = 5 experimental replicates) (**a**). Fluorescence intensity of BPGM protein in oocytes (**b**) was determined using ImageJ (Fiji) (n = 3 experimental replicates). Representative images of BPGM protein abundance following 16 h in vivo maturation (**c**–**f**) or IVM (**g**–**j**) are shown. Images were taken on a Fluoview FV10i confocal microscope (Olympus, Tokyo, Japan) using the channels DAPI (358/461 nm) (**c**, **g**), Alexa Fluor 488 for BPGM (496/519 nm) (**d**, **h**), merge of DAPI and BPGM (**e**, **i**), and bright field (**f**, **j**). Images were captured at ×60 magnification (n = 3 experimental replicates). Data are presented as mean ± SEM and analyzed using an unpaired Student's *t*-test, **: $P < 0.005$, ****: $P < 0.00005$

magnification, using imaging channels DAPI (358/461 nm), Alexa Fluor 488 for BPGM (496/519 nm), and bright field. The same imaging parameters were kept for each replicate.

Statistical analysis

Results are represented as mean ± SEM of three or more independent replicates. Statistical analyses were carried out on GraphPad Prism Version 8 for Windows (GraphPad Holdings LLC, CA, USA). Data were checked for normality and transformed accordingly. Statistical analyses were done using a one-way ANOVA or Student's *t*-tests as indicated in figure legends, and statistical significance taken at *P*-value < 0.05. Post hoc analyses were carried out when appropriate as described in figure legends.

Results

Contrasting Hb and Bpgm gene expression in mouse COCs and human cumulus cells following in vivo maturation

We first determined the in vivo level of *Bpgm* compared to known levels of Hb. Hemoglobin mRNA transcript was abundant in mouse COCs (Fig. 1 a), consistent with previously published literature [10]. When compared to the expression of the gene responsible for its partner enzyme 2,3-BPG (*Bpgm*), a contrasting pattern was observed, with significantly lower (15-fold) *Bpgm* expression compared to that of *Hba-a1* (Fig. 1 a). In human cumulus cells following in vivo maturation, the same pattern was observed: *BPGM* was significantly lower compared to *HBA* (49-fold) (Fig. 1 b).

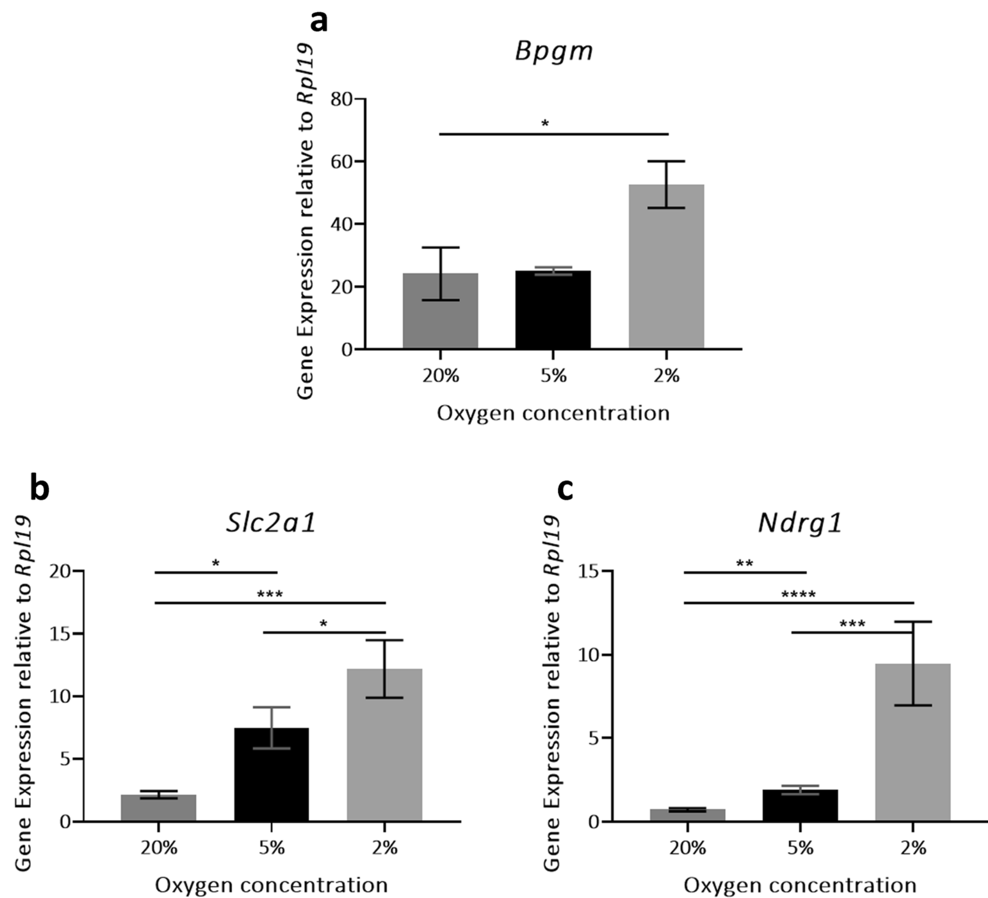

Fig. 3 Low oxygen during in vitro maturation (IVM) increased bisphosphoglycerate mutase (*Bpgm*) gene expression in cumulus-oocyte complexes (COCs) and was comparable to classic oxygen-regulated gene response. The expression of three genes were assessed in mouse COCs following 16 h IVM at 2, 5, and 20% oxygen concentrations: Bisphosphoglycerate mutase (*Bpgm*) (**a**), glucose transporter-1 ((solute carrier family 2 (facilitated glucose transporter), member 1; *Slc2a1*) (**b**), and N-Myc downstream-regulated gene 1 (*Ndrg1*) (**c**). Gene expression is presented relative to ribosomal protein L19 (*Rpl19*) and analyzed using $2^{-\Delta CT}$ (n = 3–5 experimental replicates). Data are presented as mean ± SEM and analyzed using one-way ANOVA with post hoc Tukey's multiple comparisons test. Data were log-transformed to normalize distribution prior to statistical analysis *: $P < 0.05$, **: $P < 0.005$, ***: $P < 0.0005$, ****: $P < 0.00005$

Bpgm mRNA and protein abundance in mouse COCs following in vivo and in vitro maturation

As Hb is known to be dysregulated following IVM [9], we next determined if the same was true for Bpgm. *Bpgm* was significantly higher (10.7-fold) in IVM COCs compared to in vivo–derived COCs (Fig. 2 a). The protein abundance in the oocyte was assessed following immunohistochemistry (Fig. 2 b–j), revealing a similar pattern of significantly higher abundance (1.9-fold) in IVM oocytes relative to in vivo (Fig. 2 b). Interestingly, protein abundance was higher in the oocyte compared to the cumulus cell compartment of the COC for both in vivo and IVM COCs; hence, fluorescence intensity was measured in the oocyte rather than the whole COC (Fig. 2 d, h).

Effect of oxygen concentration and exogenous Hb on Bpgm abundance

In an attempt to normalize in vitro *Bpgm* to the levels observed in vivo, two separate experimental conditions were analyzed: lowered oxygen concentration and the addition of exogenous Hb. There was a 2.2-fold increase in *Bpgm* when COCs were cultured at 2% compared to 20% oxygen (Fig. 3 a). Similar levels of expression were seen following maturation at 5% and 20% oxygen. To confirm our culture conditions sufficiently induced hypoxia, we assessed the expression of known oxygen-responsive genes *Slc2a1* and *Ndrg1*. Both *Slc2a1* and *Ndrg1* displayed similar patterns of increased expression upon exposure to 5% and 2% oxygen compared to 20% (Fig. 3 b and c, respectively), consistent with previous observations [9].

As *Bpgm* was significantly increased at 2% oxygen, we further explored whether its expression could be normalized by the addition of exogenous Hb during IVM at the same oxygen level. This was based on prior data showing the absence of Hb during IVM and abundance in vivo [10]. We expected the addition of Hb to IVM would replicate in vivo conditions and potentially normalize *Bpgm* to in vivo levels. There was a significant, 2.9-fold decrease in *Bpgm* expression in the presence of ferric Hb (Hb^{3+}) compared to that of control (Fig. 4 a). A similar reduction in *Bpgm* was seen in the presence of ferrous (Hb^{2+}) (2.0-fold) but did not reach statistical significance (P = 0.0584, Fig. 4 a). To confirm that the effect of exogenous Hb was specific to *Bpgm* and not related to changes in available oxygen, which may have been

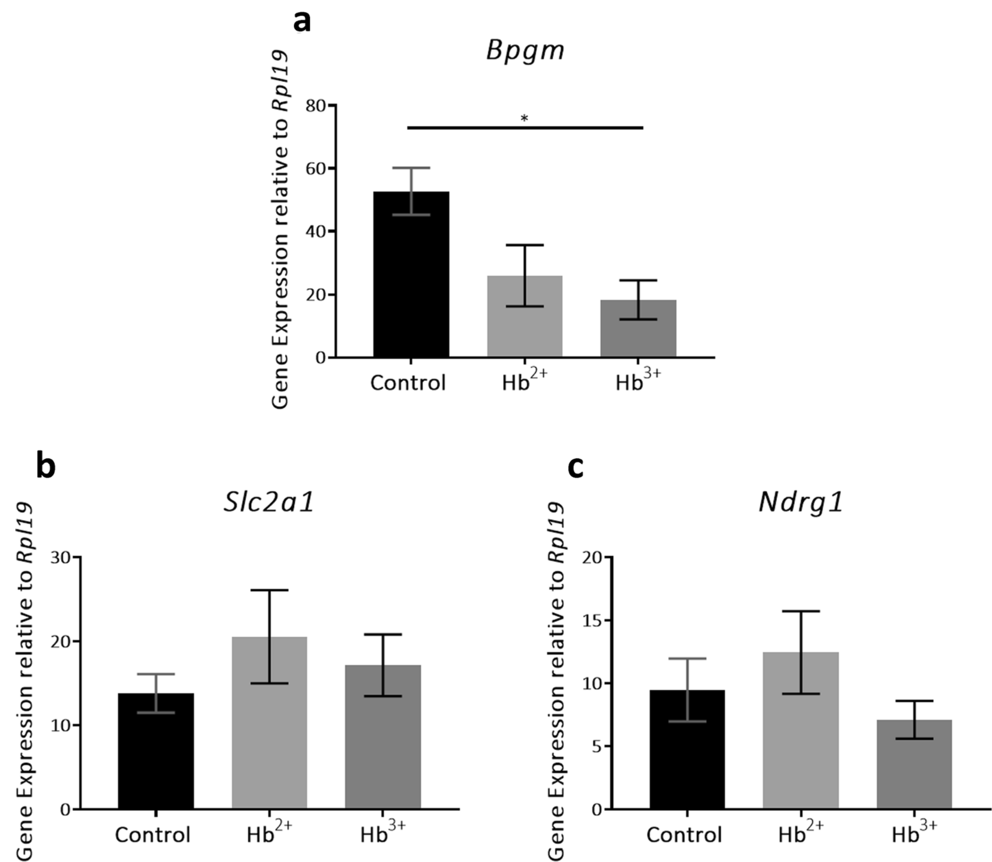

Fig. 4 Exogenous hemoglobin reduced bisphosphoglycerate mutase (*Bpgm*) gene expression in mouse cumulus-oocyte complexes (COCs) following in vitro maturation (IVM) at 2% oxygen concentration. The expression of three genes were measured in mouse COCs following 16 h IVM in the absence or presence of ferrous (Hb^{2+}) or ferric (Hb^{3+}) hemoglobin: Bisphosphoglycerate mutase (*Bpgm*) (**a**), glucose transporter-1 ((solute carrier family 2 (facilitated glucose transporter), member 1; *Slc2a1*) (**b**), and N-Myc downstream-regulated gene 1 (*Ndrg1*) (**c**). Gene expression is presented relative to ribosomal protein L19 (*Rpl19*) and analyzed using $2^{-\Delta CT}$ (n = 4–5 experimental replicates). Data are presented as mean ± SEM and log-transformed data analyzed using one-way ANOVA with post hoc Dunnett's multiple comparisons test. *: $P < 0.05$

altered through addition of Hb, we investigated the expression of oxygen-responsive genes *Slc2a1* and *Ndrg1*. The addition of Hb^{2+} or Hb^{3+} did not alter the expression of oxygen-regulated genes *Slc2a1* or *Ndrg1* (Fig. 4 b, c) confirming that the decrease in *Bpgm* was due to exogenous Hb addition alone.

In contrast to mRNA expression, the addition of exogenous Hb during IVM at 2% oxygen resulted in a visible increase in BPGM protein within the oocyte compared to that of control (Fig 5 f, j vs. b respectively). Following quantification of fluorescence intensity within the oocyte, there was a small but significant 1.3-fold increase in BPGM fluorescence in oocytes cultured in the presence of Hb^{2+} compared to control but similar levels seen in the presence of Hb^{3+} (Fig. 5 m).

Discussion

There are many non-erythroid cells found in low oxygen environments in vivo that express Hb [19], where it might play a role in regulating oxygen [20, 21]. The discovery of Hb within the in vivo matured COC [9] inspired exciting possibilities of a similar function in regulating oxygen during maturation [22]. Despite the known interaction between Hb and 2,3-BPG in red blood cell oxygen regulation [11], most non-erythroid studies have neglected to investigate the role of 2,3-BPG together with Hb. In the in vivo matured COC, Bpgm displays an opposing expression pattern to Hb during the peri-ovulatory period [10]. Similarly, *Bpgm* expression was significantly higher comparing in vitro–derived to in vivo–derived human and mouse embryos [23]. Decreasing in vitro oxygen concentration to mimic the hypoxic environment of the in vivo ovary elevates oxygen-regulated gene expression [7]. Under these conditions, a thorough investigation of Bpgm was conducted to determine its gene expression and protein abundance in COCs and attempt to normalize its expression.

In the present study, in vivo gene expression of Hb and Bpgm were directly compared, for the first time, in mouse COCs and human cumulus cells. Both models demonstrated unequivocally low levels of Bpgm compared to Hb. The characterization of Bpgm was further explored by examining how standard IVM conditions affected mRNA and protein levels, which is known to result in a significant decrease in Hb expression [10]. Interestingly, *Bpgm* expression in COCs and protein abundance within the oocyte were significantly higher in IVM COCs relative to in vivo matured COCs. This is in contrast with published levels of Hb mRNA and protein which significantly decrease following IVM [9, 10]. Furthermore, BPGM protein was predominantly localized to the oocyte following IVM. This is also in stark contrast with HBA

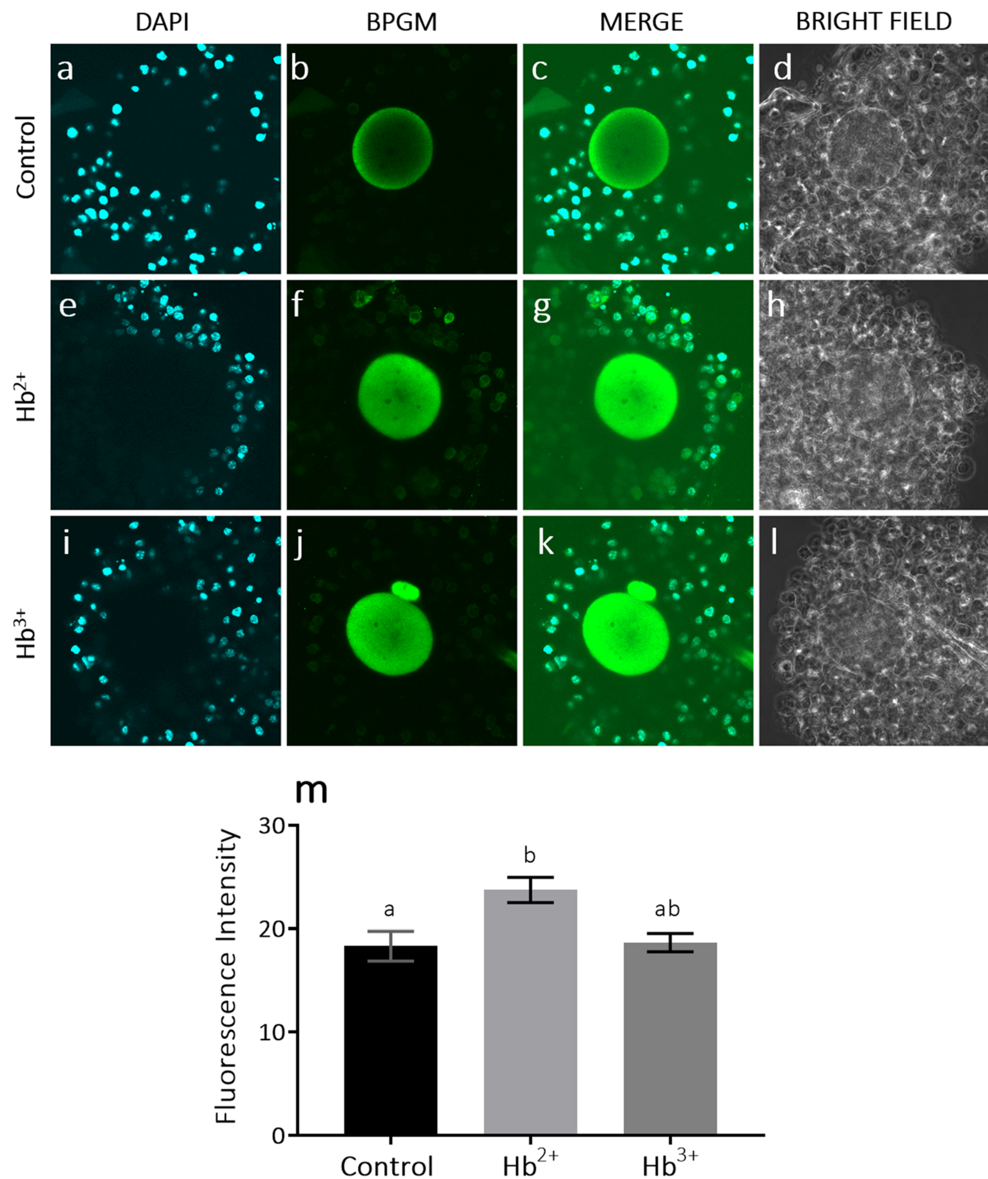

Fig. 5 Exogenous hemoglobin increased bisphosphoglycerate mutase (BPGM) protein abundance in the oocyte of cumulus-oocyte complexes (COCs) following in vitro maturation (IVM) at 2% oxygen concentration. Protein abundance of BPGM was determined using immunohistochemistry on COCs following 16 h of IVM in control conditions or following supplementation with ferrous (Hb^{2+}) or ferric (Hb^{3+}) hemoglobin. Images were taken on a Fluoview FV10i confocal microscope (Olympus, Tokyo, Japan) using the channels DAPI (358/461 nm) (**a**, **e**, **i**), Alexa Fluor 488 for BPGM (496/519 nm) (**b**, **f**, **j**), merge of DAPI and BPGM (**c**, **g**, **k**) and bright field (**d**, **h**, **l**). Images were captured at ×60 magnification (n = 3 experimental replicates). The fluorescence intensity of BPGM protein in oocytes (**m**) was calculated using ImageJ (Fiji) (n = 3 experimental replicates). Data are presented as mean ± SEM and analyzed using one-way ANOVA with post hoc Tukey's multiple comparisons test; different superscripts signify $P < 0.05$

protein which is abundant in the cumulus cells but absent from the IVM oocyte [10]. Altered gene expression is thought to be a major contributor to reduced developmental competence of in vitro matured oocytes compared to their in vivo matured counterparts [24]. This aberrant gene expression may contribute to disrupted cell growth and signaling [25]. Here, we endeavored to alter IVM conditions to resemble the in vivo environment and thus normalize *Bpgm* expression.

To mimic in vivo levels of *oxygen*, COCs were cultured at lower oxygen concentrations (2% and 5%) compared to standard IVM (20%). Two classic oxygen-regulated genes *Slc2a1* and *Ndrg1* were chosen to confirm the low oxygen culture conditions. This elicited the predicted effects on gene expression, as both genes were highly expressed at 2% and 5% oxygen concentration, validating a previous work [7]. Unexpectedly, *Bpgm* expression increased at 2% oxygen concentration compared to 20% oxygen, indicating that lowering oxygen during IVM does not normalize Bpgm to in vivo levels. The second method used to normalize *Bpgm* was exogenous addition of ferrous (Hb^{2+}) or ferric (Hb^{3+}) Hb during IVM at 2% oxygen concentration, as endogenous Hb expression remains low despite decreasing oxygen levels (Supplementary Fig. 1). The addition of Hb^{3+} significantly decreased *Bpgm* expression compared to control. This was confirmed to be due to Hb addition and not an effect of Hb on oxygen level, as there were no changes to *Slc2a1* and *Ndrg1* expression. This pattern is reminiscent of the contrasting pattern of Hb and Bpgm observed in COCs matured in vivo, which would have a lower oxygen environment close to the 2% oxygen concentration used in this experiment. This finding suggests that the presence of Hb protein was able to regulate *Bpgm* expression,

and COCs might have an inherent sensing mechanism to decrease *Bpgm* in the presence of Hb. However, BPGM protein analysis of COCs at 2% oxygen revealed that protein levels were not decreased by the addition of Hb, and instead increased when Hb^{2+} was added. A possible reason for this seemingly contradictory result is that measuring *Bpgm* mRNA at 16 h post IVM might overlook a peak in expression at an earlier stage, resulting in high levels of BPGM protein remaining present at the end of maturation. However, we conducted a time-course analysis by collecting COCs across the duration of IVM, and this showed no change in *Bpgm* at 2% oxygen but rather an initial decrease at 4 h followed by an increasing trend (Supplementary Fig. 2 a). This pattern was not altered by the presence of exogenous Hb to the culture media (Supplementary Fig. 2 b–c). These results are at odds with the in vivo COC time-course in a prior study where Bpgm was the highest at the 0 h timepoint [10], thus suggesting that culturing at 2% oxygen with exogenous Hb does not normalize Bpgm to in vivo levels.

The data described herein provides insight into the regulation of Bpgm during IVM. Future directions in this area will extend our findings to discover the in vitro conditions that best replicate the in vivo environment and normalize Bpgm and Hb levels in the COC. In the current study, we found no difference in Hb or *Bpgm* gene expression when comparing cumulus cells from women of varying age (data not shown). Future research may include determining whether levels of Bpgm and Hb in human cumulus cells are altered in patients with different characteristics or conditions (e.g. type of infertility). The characterization of Bpgm can be further interrogated using alternative molecular techniques. While the current study utilized immunofluorescence, a western blot for Bpgm would be informative and confirm our results. It would be of further benefit to quantify the levels of 2,3-BPG produced by Bpgm and study its interaction with Hb. However, this is hindered by the lack of a specific antibody for 2,3-BPG.

One important point made clear in our work is that while lowering oxygen concentration induces oxygen-regulated gene expression in the in vitro COC, it did not restore Hb and Bpgm to in vivo levels. Additionally, as prior work has shown Hb to be present in granulosa cells [10], future in vitro studies could consider utilizing a follicle-like coculture system that includes granulosa cells, which may restore Hb in the COC to levels observed in vivo. Alternatively, where future studies employ exogenous Hb during IVM, the efficiency of Hb uptake by COCs would be insightful. From the current work, it appears that exogenous Hb might not behave in the same way as endogenous Hb found in vivo. This is evident in comparing our in vitro time-course of *Bpgm* expression in the presence of exogenous Hb with the in vivo counterpart from prior work [10].

In summary, our work offers further insight into the potential role of Hb in oxygen-regulated gene expression, through the characterization of Bpgm in the in vivo and in vitro matured COC. We show that the in vitro culture environment results in dysregulation of Bpgm that cannot be normalized by hypoxic conditions or exogenous Hb. Discovery of the in vitro conditions that normalize the levels of Bpgm and Hb in the COC will be best positioned to investigate the impact on oocyte developmental competence.

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Author contribution ML performed the experiments, analyzed data, wrote and edited the manuscript. HMB, RDR, and JGT provided critical feedback on the final manuscript. JGT conceived the original idea. KRD supervised the project, conceived and planned the experiments, and aided in interpreting results and in editing of the manuscript.

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Data availability All data generated or analyzed during this study are included in this published article and are available from the corresponding author on reasonable request.

Code availability N/A

Declarations

Ethics approval Ethics approval for the study was obtained from the Human Research Ethics Committee, University of Adelaide, Adelaide, Australia (approval number H-2018-205) and the University of Adelaide Animal Ethics Committee (M-2016-147).

Consent to participate Consent for cumulus cells to be stored and used for research purposes (St Andrews Hospital, Human Research Ethics Committee Project No. 93).

Consent for publication The authors consent for publication of this article.

Conflict of interest The authors declare no competing interests.

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