Oxygen-regulated gene expression in murine cumulus cells.

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Abstract

Oxygen is an important component of the environment of the cumulus-oocyte-complex (COC), both *in vivo* within the ovarian follicle and during *in vitro* oocyte maturation (IVM). Cumulus cells have a key role in supporting oocyte development, and cumulus cell function and gene expression is known to be altered when the environment of the COC is perturbed. Oxygen regulated gene expression is mediated through the actions of the transcription factors, the Hypoxia inducible factors (HIFs). In the current study, the effect of oxygen on cumulus cell gene expression was examined following *in vitro* maturation of the murine COC at 2%, 5% or 20% oxygen. Increased expression of HIF-responsive genes, including glucose transporter-1, lactate dehydrogenase A, and BCL2/adenovirus E1B interacting protein-1 NIP3, was observed in cumulus cells matured at 2% or 5%, compared to 20% oxygen. Stabilisation of HIF1α protein in cumulus cells exposed to low oxygen was confirmed by Western blot and HIF mediated transcriptional activity was demonstrated using a transgenic mouse expressing green fluorescent protein under the control of a promoter containing hypoxia response elements. These results indicate that oxygen concentration influences cumulus cell gene expression and support a role for HIF1α in mediating the cumulus cell response to varying oxygen.

Key words: cumulus cells / hypoxia inducible factors / oocyte / oxygen

Running Title: Oxygen regulated gene expression in cumulus cells

Introduction

Oxygen is an important component of the environment during oocyte maturation, both *in vivo* and *in vitro*. Mathematical modelling suggests that dissolved oxygen levels in human follicular fluid during the late antral to preovulatory stage are low and range between 11 and 51 mm Hg (1.5 – 6.7 %) (Redding *et al*. 2008). Although studies directly measuring oxygen content in aspirated follicular fluid report varying results, several studies agree with the modelling estimates, and report oxygen levels of 1-5% (Van Blerkom *et al*. 1997; Huey *et al*. 1999; Redding *et al*. 2008). However, oxygen contents at the low end of this range have been associated with reduced oocyte developmental competence, with reduced development rates and increased chromosomal abnormalities reported in embryos developed from oocytes exposed to follicle oxygen contents of less than 1.5% (Van Blerkom *et al*. 1997).

The use of a physiologically relevant concentration of 5-7% oxygen has been clearly shown to enhance *in vitro* embryo development and quality (Thompson *et al*. 1990; Gardner *et al*. 1996; Meintjes *et al*. 2009; Waldenstrom *et al*. 2009; Wale *et al*. 2010; Bontekoe *et al*. 2012); however, the optimal oxygen concentration for oocyte maturation *in vitro* is less well defined. While 5% oxygen has been reported to have negative effects on nuclear maturation of bovine oocytes, when compared to 20% oxygen (Pinyopummintr and Bavister, 1995), others report improved developmental competence following maturation of bovine oocytes at 5% oxygen (Hashimoto *et al*. 2000; Hashimoto, 2009; Bermejo-Alvarez *et al*. 2010; Pereira *et al*. 2010). Media composition may contribute to this variation, as Hashimoto *et al*. (2000) reported that 5% O_2 has beneficial effects on bovine oocyte meiotic maturation at glucose concentrations of 20 mM, but not 1.5 mM. In mice, early studies

reported improved nuclear maturation rates when IVM was performed at 5%, compared to 20% oxygen (Haidri *et al*. 1971). Others have reported no differences in blastocyst development, but an increase in blastocyst cell numbers, following maturation of mouse oocytes at 5%, compared to 20% oxygen (Preis *et al*. 2007). Similarly, trophectoderm cell numbers decreased in mouse blastocysts as oxygen concentration used during IVM increased, across a range from 2% to 20% oxygen (Banwell *et al*. 2007). Trophectoderm cell numbers were highest following maturation of mouse oocytes at 2%, compared to 5%, 10% or 20% oxygen (Banwell *et al*. 2007). Oocyte maturation at 5% oxygen was associated with reductions in the subsequent fetal and placental weight, when compared to 20% oxygen, or *in vivo* oocyte maturation respectively (Banwell *et al*. 2007). These results suggest that the effects of varying oxygen concentrations, across a wider range, on cumulus-oocyte-complex (COC) function require further analyses.

Culture conditions used during IVM influence both the health and function of the oocyte and its surrounding cumulus cells. The cumulus cells have an important role in supporting oocyte development, through provision of nutrients, energy substrates and ions via gap-junctional communication (Eppig 1991; Albertini *et al*. 2001). While the fully grown oocyte is largely dependent on oxidative phosphorylation for ATP production, glucose metabolism by cumulus cells is a significant source of energy substrates, such as pyruvate, for metabolism by the oocyte (Sutton-McDowall *et al*. 2010). Furthermore, it has been demonstrated that cumulus cells consume relatively little oxygen, sparing it for the oocyte (Clark *et al*. 2006). Therefore, the metabolic activity of the oocyte and cumulus cells varies significantly, suggesting that the specific oxygen requirements of the oocyte and cumulus cell may also vary. The effects of varying oxygen concentration during IVM on cumulus cell function should also, therefore, be considered. The gene expression profile of cumulus

cells is commonly associated with oocyte developmental competence and embryo viability (Van Montfoort *et al*. 2008; Assou *et al*. 2010; Gebhardt *et al*. 2011). Altered expression of metabolic genes in bovine cumulus cells has been reported following maturation at 5% compared to 20% oxygen (Bermejo-Alvarez *et al*., 2010), suggesting oxygen mediated gene expression as a potential mechanism through which oxygen could influence COC function.

Therefore, in the current study, gene expression in murine cumulus cells was assessed following *in vitro* maturation at a range of oxygen concentrations, from 2% to 20%. Initially we conducted preliminary microarray analyses which identified that genes known to be regulated by the hypoxia-inducible factors (HIFs) were increased in murine cumulus cells exposed to low oxygen conditions. HIFs are transcription factors that mediate the response to low oxygen (Semenza, 2000; Wenger, 2002; Semenza, 2010). Previous studies have reported that HIF1α is associated with follicular differentiation, is expressed in granulosa and luteal cells, and is induced in granulosa cells around the time of ovulation (Duncan *et al*., 2008; Kim *et al*., 2009; Tam *et al*., 2010), but a role for HIF 1α in cumulus cells has not been assessed. Therefore, the current study also aimed to determine whether oxygen dependent regulation of gene expression in cumulus cells is associated with HIF1α protein stabilisation.

Materials and Methods

Cumulus-oocyte-complex collection and in vitro maturation

All experiments were conducted according to the National Health and Medical Research Council of Australia guidelines and were approved by The University of Adelaide Animal Ethics Committee. Unless otherwise specified, all chemicals and reagents were purchased from Sigma Chemical Co. (St Louis, USA). COCs were isolated from 23-25 day old female hybrid CBAB6F1 mice. Mice were injected with 5 IU equine chorionic gonadotrophin (eCG) (Folligon serum gonadotropin; Intervet, Merck Animal Health, Boxmeer, Holland) 44 - 46 h prior to COC collection. COCs were collected into HEPES buffered αMEM media (Invitrogen, Life Technologies, Carlsbad, USA) supplemented with 50 μg/ml streptomycin sulphate, 75 μg/ml penicillin G and 5% fetal bovine serum (FBS) (Invitrogen, Life Technologies) by gently puncturing visible antral follicles on the ovary surface with a 30 gauge needle. COCs containing a germinal vesicle stage oocyte with an intact vestment of cumulus cells were matured (10 per drop) in 100 μl drops of bicarbonate buffered αMEM supplemented with 50 μg/ml Streptomycin, 75 μg/ml Penicillin G, 5% FBS and 50 mIU/ml recombinant human follicle stimulating hormone (rhFSH) (Puregon; Organon, Sydney, Australia) under oil in 35-mm Falcon 1008 culture dishes (Becton-Dickinson Labware, Franklin Lakes, USA). COCs were matured for 17 h under gas mixes of 2, 5 or 20% oxygen (6% carbon dioxide and balance of nitrogen) at 37^{o}C in modular incubation chambers (Billups-Rothenburg, Del Mar, USA) filled with test gas mixtures (Banwell *et al*., 2007).

Cumulus cell collection for gene expression analyses

Following 17 h IVM, COCs from each treatment group (2, 5 or 20% oxygen) were transferred into 150 µl drops of HEPES buffered αMEM containing 25 U/ml ovine hyaluronidase and all cumulus cells were dissociated with the aid of gentle pipetting with a fine glass pipette. Oocytes were removed and the remaining cumulus cells were collected into 20 µl of medium in sterile, 1.5 ml tubes. Cumulus cells were then washed in fresh HEPES buffered αMEM twice and spun down at 16 000 *g* for 2 min before being frozen in liquid nitrogen and stored at -80^{o}C until use.

For analysis of gene expression, COC collections were performed from 12 mice to generate pools of cumulus cells from 70-120 *in vitro* matured COCs for each of the 3 oxygen concentrations. These collections were repeated on 5 separate occasions to generate 5 pools of cells from each oxygen concentration for RNA extraction for real-time RT-PCR analysis.

Cumulus cell gene expression analyses

Total RNA was isolated from the cumulus cell samples using the RNeasy Micro Kit (Qiagen, Doncaster, USA) according to the manufacturer's instructions. Genomic DNA was removed by DNase treatment as per the kit instructions. RNA samples were stored at -80^{o}C.

Genes for analysis were chosen based on results of a preliminary microarray analysis (n=1 array, utilising RNA generated from cumulus cells matured at 2%, 5%, 10% or 20% oxygen; data not shown). Two sets of genes were selected for quantitative gene expression by real-time RT-PCR (qPCR). The preliminary microarray analysis indicated up-regulation

of a number of expressed probes in cumulus cells matured at reduced oxygen, when compared to 20% oxygen (2% oxygen, 55 up-regulated; 5% oxygen, 31 up-regulated; 10% oxygen, 15 up-regulated). Up-regulated genes included genes known to be regulated by the hypoxia-inducible factors (HIFs). A selection of these genes suggested by preliminary microarray analysis as increased 6-9 fold following exposure to 2% oxygen were chosen for analysis. Expression of glucose transporter-1 *(Slc2a1)*, lactate dehydrogenase A (*LdhA*), enolase 1 (*Eno1*), phosphoglycerate kinase (*Pgk1*), N-myc downstream regulated gene 1 (*Ndrg1*), and BCL2/adenovirus E1B interacting protein 1 NIP3 (*Bnip3*) was analysed. Hypoxia inducible factor-1α (*Hif1α)* was indicated as down-regulated and was also analysed. Preliminary microarray analysis also indicated down-regulation of a range of other genes in cumulus cells at reduced oxygen (2% oxygen, 218 down-regulated; 5% oxygen, 305 down-regulated). This included a number of genes involved in lipid metabolism. Down-regulated expression of metabolic genes in response to low oxygen was of interest, and a selection of these genes indicated by microarray as decreased by 3-6 fold following exposure to 2% oxygen were chosen for analysis. Expression of ELOVL family member 6, elongation of long chain fatty acids (*Elovl6*), inositol polyphosphate-5-phosphatase E (*Inpp5E*), acyl-CoA synthetase short-chain family member 2 (*Acss2*), stearoyl-Coenzyme A desaturase 1 (*Scd1*) and Trans-2-enoyl-CoA reductase, mitochondrial (*Mecr*) was analysed.

The effect of IVM oxygen concentration on mRNA abundance of the genes of interest in cumulus cells was analysed by qPCR using an ABI PRISM® 5700 sequence detection system (Applied Biosystems, Foster City, CA, USA). RNA was extracted from the cumulus cell samples collected as described above (n = 5 pooled samples per treatment).

Total RNA (500 ng) was reverse transcribed using random primers and Superscript III Reverse transcriptase (200 units) (Invitrogen, Life Technologies).

Oligonucleotide primers were designed using Primer Express (Applied Biosystems) and were synthesised by GeneWorks, Adelaide, Australia. Primer sequence details are described in Table 1. PCR was performed in 20 μl volumes containing 1x SyBr Green Master Mix (Applied Biosystems) and 500 nM forward and reverse primers.

cDNA equivalent to 10 ng RNA was analysed in duplicate for each gene of interest. For *18S* rRNA, cDNA equivalent to 2.5 ng RNA was analysed. The thermal cycling program consisted of 2 min at 50^{o}C, 10 min at 95^{o}C, followed by 40 cycles of 15 sec at 95^{o}C and 1 min at 60^{o}C. A quantification cycle (Cq) was calculated for each sample using the GeneAmp 5700 software. Dissociation curves and no template samples were used to confirm the absence of non-specific amplification products. Standard curves were generated using cDNA generated from whole mouse ovary. Curves were plotted as log starting RNA concentration versus Cq for each standard, and expression in each experimental sample, relative to the level of expression in the ovary standard, was calculated from the standard curve. Results were normalised against those obtained for the reference genes mitochondrial ribosomal protein L19 (*Mrpl19*) (Dunning *et al*. 2007) and *18S* rRNA. All results were then expressed as a fold change from the group matured at 20% oxygen.

Western immunoblotting

COCs were collected from 12 mice and matured at 2%, 5% or 20% oxygen for 17 h, as described above, and HIF1α protein was analysed by Western blot as previously described (Tam *et al*. 2010). At the completion of IVM, COCs (100 per treatment) were rapidly

washed in protein free αMEM. COCs were lysed in RIPA buffer (Sigma Aldrich) in the presence of protease inhibitors (Protease Inhibitor Cocktail P8340, Sigma Aldrich), frozen in liquid nitrogen and stored at -80^{o}C. Three replicate experiments were performed. Whole COCs were collected, rather than cumulus cells, as initial experiments indicated that the time taken to separate cumulus cells from the oocyte resulted in some degradation of the HIF protein. To confirm whether the source of the immunoreactive bands detected in the Western was the cumulus cells or oocyte, additional experiments were performed with maturation of COCs at 5% oxygen, with addition of cobalt chloride. Cobalt chloride, a hypoxia mimetic, stabilises the HIF1α protein by inhibiting the activity of the prolyl hydroxylases that target HIF1α for degradation. COCs (n=100) were matured in the presence of 250 μM cobalt chloride for the final 4 h of IVM. Cumulus cells from the cobalt chloride treated COCs were removed by addition of hyaluronidase, as described above, and separated cumulus cells and oocytes were lysed in RIPA buffer containing protease inhibitors, frozen in liquid nitrogen and stored at -80^{o}C.

Additional experiments examined HIF1α protein expression in *in vivo* matured and immature COCs. Twelve mice were injected with eCG as described above. From 8 of the mice, COCs were collected at 44-46 h post-eCG injection into HEPES buffered αMEM. COCs containing a germinal vesicle stage oocyte and intact vestment of cumulus cells from 4 of these mice were immediately lysed in RIPA buffer containing protease inhibitors, frozen in liquid nitrogen and stored at -80^{o}C (immature, germinal vesicle stage COCs). COCs from the remaining 4 mice were matured *in vitro* at 5% oxygen for 17 h. COCs were then lysed and frozen as described above (*in vitro* matured). The remaining 4 mice were injected with 5 IU hCG 48 h post-eCG injection. COCs were flushed from the oviduct 17 h

198 post-hCG injection into HEPES buffered αMEM, lysed and frozen as described above (*in*
199 *vivo* matured). Two replicate experiments were performed.

200 Proteins were separated by SDS-PAGE through a 4% stacking and 10% separating gel and
201 transferred onto PVDF membranes. All gels for Western blotting included pre-stained
202 protein molecular weight markers (Bio-Rad, Hercules, CA, USA). Membranes were
203 blocked for 1 h with 5% (w/v) skim milk, 1x TBST (10 mM Tris-HCl (pH 8.0), 150 mM
204 NaCl and 0.05% Tween 20). Detection of target proteins was accomplished using a rabbit
205 polyclonal antibody specific for HIF1α (1:1000, NB100-449, Novus Biologicals, Littleton,
206 CO, USA) followed by a goat anti-rabbit secondary antibody conjugated to HRP (Santa
207 Cruz Biotechnology, Santa Cruz, CA, USA) at 1:5000 dilution in 5% (w/v) non-fat milk,
208 TBST and 0.05% Tween 20 for 1 h and detection by enhanced chemiluminescence as per
209 the manufacturer's instructions (Amersham, GE Healthcare Life Sciences, Rydalmere,
210 NSW, Australia). The antibodies were removed with acidic glycine stripping buffer (1%
211 SDS, 25 mM glycine, pH 2) and the membranes were incubated overnight at 4^{o}C with a
212 mouse monoclonal antibody to β-actin (Sigma Aldrich) at a dilution of 1:25 000. This was
213 followed by incubation with a goat ant-mouse secondary antibody conjugated to HRP and
214 detection by enhanced chemiluminescence, as described above. Mammary tumour from a
215 MMTV-PyMT mouse was used as a positive control sample in the Western blots. For
216 extraction of protein the tumour sample was homogenised (Precellys Homogeniser, Bertin
217 Technologies, Montigny-le-Bretonneux, France) in RIPA buffer containing protease
218 inhibitors.

219 *HRE-EGFP transgenic reporter mouse*

COCs were also collected from the ovaries of Hypoxia response element (HRE)-EGFP transgenic reporter mice (Tam *et al*. 2010). The transgenic mouse contains a HRE-EGFP construct, developed using a previously described pSV40 promoter-EpoHRE-Luc plasmid (Ema *et al*. 1996). The plasmid contained four HREs of the *Epo* gene (coding strand, 5′-GATCGCCCTACGTGCTGTCTCA-3′) inserted in tandem into the BglII site of pGL3 promoter plasmid (Promega, Madison, WI, USA) (Ema *et al*., 1996). An Xba I/Hind III fragment from the pEGFP-N1 vector (Clontech, Mountain View, CA, USA), containing the coding sequence for EGFP, was inserted into the plasmid in place of the gene encoding luciferase. A lentiviral vector containing the HRE(4)-SV40-EGFP sequence was then generated by Ozgene Pty Ltd, and C57BL/6 transgenic mice (C57BL/6-Tg(HRE(4)-SV40-EGFP) were produced by lentiviral incorporation of the vector (Ozgene, Bentley, WA, Australia). COCs were collected from four 21-28 day old HRE-EGFP transgenic mice 44 h post injection with eCG, and matured *in vitro* at 2% or 20% oxygen. An additional HRE-EGFP positive mouse was injected with 5 IU hCG, 46 h post eCG, and *in vivo* matured COCs were flushed from the oviduct 20 h post-hCG. COCs from a mouse that tested negative for the presence of the transgene were also collected and *in vitro* matured at 2% oxygen as a negative control.

Following 17 h IVM at either 2% or 20% oxygen, or *in vivo* maturation, COCs were transferred to a 10 µL drop of HEPES-buffered αMEM medium in glass bottomed petri dishes for live cell imaging (Cell E&G, Houston, TX USA) for fluorescent imaging. Images were examined using a Confocal laser scanning microscope with a 473 nm diode laser (Olympus Fluoview, FV10i, Olympus Corporation, Tokyo, Japan). Fluorescent emission was collected at 510 nm. The images were captured at 40x magnification and

processed using the Olympus FV10i-SW software and camera (Olympus Corporation, Tokyo, Japan).

Statistical Analyses

Statistical significance was determined using SPSS software for Windows, version 14.0 (SPSS, Chicago, IL, USA). Cumulus cell gene expression results were analysed using one-way analysis of variance followed by least-significant difference (LSD) post-hoc test. Statistical analysis was performed following normalisation to the reference gene. The data are presented as the mean ± SEM. Significance was accepted at a P value of < 0.05.

Results

Effects of Oxygen on Cumulus Cell Gene Expression

The effect of *in vitro* maturation under 2%, 5% or 20% oxygen on cumulus cell expression of selected genes of interest was confirmed by qPCR (Figures 1 and 2). Results are shown normalised against expression of the housekeeper gene mitochondrial *Rpl19*. Normalisation against *18S rRNA* was also performed with similar results (data not shown).

Slc2a1, Ldha, Eno1, Pgk1, Bnip3 and *Ndrg1* mRNA levels in cumulus cells were significantly up-regulated following *in vitro* maturation at 2% or 5% when compared to 20% O_2 (Figure 1). Higher expression of *Slc2a1*, *Bnip3* and *Ndrg1* mRNA was also seen in cumulus cells cultured under 2% O_2 when compared to 5% O_2 (Figure 1, $P < 0.03$). In contrast to the HIF target genes, *Hif1α* mRNA abundance was reduced in cumulus cells matured at 2% O_2, when compared to 20% O_2 (Figure 1).

263 The mRNA abundance of *Mecr, Scd1, Elovl6, Inpp5e* and *Acss2,* genes involved in lipid
264 metabolism and suggested by a preliminary microarray to be down-regulated (by 3-6 fold)
265 in cumulus cells following exposure to 2% oxygen (data not shown), was also assessed
266 (Figure 2). Oxygen concentration did not significantly alter expression of *Mecr* and *Inpp5e*
267 in cumulus cells. However, *Elvol6* mRNA abundance decreased in cumulus cells matured
268 at 2% O_2, compared to 5% or 20% O_2 (Figure 2; $P < 0.02$). *Scd1* mRNA in cumulus cells
269 was decreased following IVM in 2% O_2 when compared to 20% O_2 (Figure 2; $P < 0.01$).
270 *Accs2* expression in cumulus cells was variable and a non-significant trend to decrease with
271 lower oxygen was observed.

272 *HIF1α protein in cumulus cells*

273 To confirm the presence of HIF1α protein in cumulus cells, COCs were treated for 4 h with
274 250 μM $CoCl_2$, and cumulus cells and oocytes were separated and analysed by Western
275 blot (Figure 3a). HIF1α protein was detected at 120 kDa in $CoCl_2$ treated cumulus cells
276 (Figure 3a). No 120 kDa HIF1α protein band was detected in $CoCl_2$ treated oocytes
277 (Figure 3a). This suggests that 120 kDa HIF1α protein detected in whole COCs represents
278 cumulus cell HIF1α (Figure 3b). HIF-1α was present at 120 kDa in whole COCs matured
279 at 2% or 5% oxygen (Figure 3b). Decreased HIF-1α protein expression was observed in
280 COCs matured at 20% oxygen. A higher molecular weight band (150 kDa) was also
281 detected in all samples. Immunoprecipitation and proteomic analysis of this band (data not
282 shown) was not able to identify the protein, suggesting that the band may represent non-
283 specific binding of the antibody.

HIF1α protein expression was additionally examined in immature, germinal vesicle stage COCs, collected by puncture of antral follicles 44 h post-eCG, in COCs that had been matured *in vitro* at 5% O_2 for 17 h, and in *in vivo* matured COCs, flushed from the reproductive tract 17 h post injection of hCG (Figure 3c). HIF1α protein at 120 kDa was detected in immature and IVM COCs, but was decreased in *in vivo* matured, ovulated (IVV) COCs.

Further confirmation of HIF activity in cumulus cells was demonstrated by observation of the presence of EGFP protein in cumulus cells of COCs collected from the HRE-EGFP reporter mouse and matured at 2% oxygen (Figure 4). EGFP was evident in cumulus cells of COCs matured at 2% oxygen (Figure 4a), when compared to COCs matured at 20% oxygen (Figure 4b) or COCs matured *in vivo* and flushed from the reproductive tract (Figure 4c).

Discussion

The oxygen concentration used during *in vitro* maturation of the murine COC had a significant effect on cumulus cell gene expression in the current study. Cumulus cell expression of genes known to be regulated by the HIF transcription factors was increased when maturation was performed at 2% or 5% oxygen, compared to 20% oxygen. The presence of HIF1α protein in cumulus cells was confirmed by Western blot, and HIF activation of gene expression was indicated by EGFP production in cumulus cells of HRE-EGFP transgenic reporter mice. Thus, these results identify oxygen-regulated, HIF mediated gene expression in cumulus cells, suggesting the potential for effects of the oxygen environment on cumulus cell metabolism and function.

306 The HIF responsive genes *Slc2a1, Ldha, Eno1, Pgk1, Bnip3* and *Ndrg1* were up-regulated
307 in cumulus cells following IVM at 2% and 5% oxygen, compared to 20% oxygen.
308 Increased expression of *Slc2a1, Ldha, Eno1* and *Pgk*, suggests that glucose uptake and
309 glycolysis are increased in cumulus cells when oxygen levels are reduced. In agreement,
310 others have reported increased expression of *SLC2A1, GAPDH and LDHA* in bovine
311 cumulus cells, following maturation under 5% oxygen, compared to 20% oxygen
312 (Bermejo-Alvarez *et al*. 2010). Cumulus cells are known to account for the majority of
313 glucose taken up by the COC; with processing via the glycolytic pathway, and production
314 of pyruvate and lactate for provision to the oocyte accounting for a significant proportion of
315 cumulus cell glucose metabolism (Thompson *et al*. 2007; Sutton-McDowall *et al*. 2010).
316 HIF mediated expression of glucose transporters and glycolytic enzymes has been well
317 described in many cells and tissues (Semenza 2010). Increased glucose uptake and lactate
318 production have been reported in mouse COCs matured in the absence of EGF under 5%,
319 compared to 20% oxygen; however, no differences were observed when EGF was included
320 in the maturation media (Preis *et al*. 2007). Beneficial effects of low oxygen on maturation
321 of bovine oocytes are dependent on supply of adequate glucose in the media, supporting the
322 importance of altered COC metabolism (Hashimoto *et al*. 2000). The extent to which
323 oxygen concentration during IVM influences metabolic activity of the cumulus cells, and
324 the potential for interactive effects of media composition, including glucose availability, on
325 cumulus cell gene expression, therefore requires further study.

326 *Bnip3* was also higher in cumulus cells matured at low oxygen. This gene belongs to the
327 NIP3 protein family, is a member of the Bcl-2 family and predominantly localises to the
328 mitochondria (Chen *et al*. 1997; Mellor and Harris 2007). *Bnip3* is significantly up-
329 regulated in response to hypoxia in many cell lines (Bruick 2000; Mellor and Harris 2007;

Bellot *et al*. 2009). However, while BNIP3 is generally defined as a proapoptotic factor, recent studies also suggest that it may contribute to a cell survival response under conditions of moderate hypoxia, by inducing mitochondrial autophagy, thus limiting the production of reactive oxygen species (Zhang *et al*. 2008; Bellot *et al*. 2009; Mazure and Pouyssegur 2010). This suggests mitochondrial number or function in cumulus cells following maturation at low oxygen as a potential area for further analysis.

Expression of *Ndrg1* was also significantly increased in cumulus cells matured at 2% or 5% oxygen, compared to 20% oxygen. This gene is a member of the N-myc downstream regulated gene family and encodes a predominantly cytoplasmic protein (Ellen *et al*. 2008; Melotte *et al*. 2010). Expression of *Nrdg1* is up-regulated in response to various cell stress conditions, including hypoxia (Salnikow *et al*. 2002; Ellen *et al*. 2008). Similarly, exposure to iron chelators, which act as hypoxia mimetics, increases *Nrdg1* expression (Melotte *et al*. 2010; Chen *et al*. 2012). However, both HIF dependent and independent pathways have been identified in the hypoxic regulation of *Ndrg1* expression (Ellen *et al*. 2008; Melotte *et al*. 2010). A range of actions of the protein have been described, including involvement in the DNA damage response, cell stress response, cell proliferation and differentiation, inhibition of cell migration, invasion and the epithelial-mesenchymal transition (Ellen *et al*. 2008; Melotte *et al*. 2010; Chen *et al*. 2012). Contrasting associations of *Nrdg1* expression with either tumour progression or suppression have been reported, suggesting tissue and cancer type specific effects (Melotte *et al*. 2010). In human trophoblasts, *Nrdg1* is up-regulated by hypoxia, where it appears to interact with the p53 signalling pathway to attenuate hypoxic injury (Chen *et al*. 2006). Up-regulation of *Nrdg1* in cumulus cells *in vitro* at 2% and 5% oxygen, when compared to 20% oxygen, suggests that the cells may be activating physiological stress protective mechanisms under these conditions.

354 Five genes involved in lipid metabolism that were suggested by a preliminary microarray as
355 having a lower expression in cumulus cells matured at 2% or 5% were also chosen for
356 analysis. Quantitative PCR determined that cumulus cell expression of 2 of the genes
357 (*Elovl6* and *Scd1*) was down-regulated at 2% oxygen and the remaining 3 genes showed no
358 statistically significant differences in expression across the varying oxygen treatments
359 during IVM. Elongation of long-chain fatty acids family member 6 (*Elovl6*) belongs to a
360 family of endoplasmic reticulum enzymes, and is involved in the elongation of saturated
361 fatty acids with 12-16 carbons to C18 (Jakobsson *et al*. 2006; Matsuzaka and Shimano
362 2006). Stearoyl-CoA desaturase 1 (*Scd1*) is also crucial in lipid biosynthesis (Flowers and
363 Ntambi 2008; Igal 2010). SCD catalyses the synthesis of monounsaturated fatty acids from
364 saturated fatty acids (Flowers and Ntambi 2008). Cardiomyocyte specific expression of an
365 oxygen stable HIF1α has been associated with decreased *Scd1* expression in mouse hearts.
366 Similarly, hepatic expression of *Scd1* is decreased in mice with liver specific
367 overexpression of HIF2 (Rankin *et al*. 2009). In contrast, macrophages exposed *in vitro* to
368 1% oxygen increased *Scd1* expression (Bostrom *et al*. 2006) and mice exposed to
369 intermittent hypoxia showed an up-regulation of hepatic SCD-1 (Li *et al*. 2006), possibly
370 due to HIF mediated stimulation of SREBP-1. Thus the effects of low oxygen on *Scd1*
371 expression appear to be cell and tissue specific, and the extent to which these effects are
372 mediated by low oxygen or HIF proteins in cumulus cells, or are downstream of other
373 metabolic changes would require further analysis.

374 Significant up-regulation of genes involved in the physiological response to low oxygen
375 supports a role for the HIF transcription factors in mediating this molecular response in
376 cumulus cells. The HIF proteins regulate gene expression by binding to the hypoxia
377 response element in the promoter region of target genes (Wenger 2002; Hopfl *et al*, 2004;

Semenza 2006; Semenza 2010). HIF1 is a heterodimeric protein, consisting of HIF1α and HIF1β subunits. Stability of the HIF1α subunit is regulated by oxygen, with the protein being protected from proteosomal degradation under low oxygen conditions (Wenger 2002; Semenza 2010). Although hypoxia is considered to be the major inducer of HIF1, non-hypoxic factors, including hormones, growth factors and inflammatory cytokines, can also induce HIF1 mediated responses in normoxic cells (Bilton and Booker 2003; Dery *et al*, 2005; Dehne and Brune 2009; Pringle *et al*, 2010). Within the murine ovarian follicle, HIF1α has been detected in granulosa cells of the preovulatory follicle, with highest levels occurring around the time of ovulation (Kim *et al*. 2009; Tam *et al*. 2010). Hormonal regulation, through LH, acting in synergy with low oxygen conditions is thought to contribute to HIF1α stabilisation in granulosa cells (Tam *et al*. 2010). In the current study, the presence of HIF1α in an additional follicle cell type, the cumulus cells, was confirmed *in vitro*. Levels of HIF-1α protein were highest in cumulus cells exposed to low oxygen, when compared to 20% oxygen, supporting a role for HIF1α in regulating oxygen mediated gene expression in cumulus cells *in vitro*, at both 2% and 5% oxygen. Similarly, when COCs from HRE-EGFP reporter mice were matured at 2% oxygen, EGFP was evident in cumulus cells, suggesting activation of HIF protein. *Hif1α* mRNA in cumulus cells was decreased following exposure to 2% oxygen. This is consistent with primary regulation of HIF1α by oxygen occurring at the level of protein stabilisation (Semenza, 2010), whereby degradation of HIF proteins is reduced under conditions associated with HIF activation. Down-regulation of *Hif1α* mRNA with chronic hypoxia is known to occur, representing an adaptive response to prolonged hypoxia (Gradin *et al*. 1996; Uchida *et al*. 2004). A similar response was observed in cumulus cells following 17 h exposure to low oxygen, and this

401 also suggests that a greater increase in HIF1α protein levels may have been observed at
402 earlier time-points.

403 HIF1α protein was also evident in immature COCs collected directly from antral follicles.
404 Our previous studies have detected HIF1α protein in granulosa cells collected 44 hours
405 post-eCG, with levels increasing post-hCG injection and peaking at ovulation (Tam *et al*.
406 2010). Similarly, HIF-mediated EGFP expression was maximally induced in granulosa
407 cells around the time of ovulation (Tam *et al*. 2010). Further analyses are required to more
408 fully examine HIF1α stabilisation in cumulus cells *in vivo*, and how this relates to follicular
409 oxygen content and hormonally regulated follicle maturation. In contrast, HIF1α was
410 barely detectable in ovulated COCs flushed from the oviduct. Whether HIF protein is
411 present in ovulated COCs earlier than 17 h post-hCG or whether the handling procedures
412 and time associated with collection of *in vivo* matured COCs from the oviduct may have
413 resulted in a degradation of HIF protein requires further study. However, EGFP was
414 similarly not detected within *in vivo* matured COCs collected from the oviduct of HRE-
415 EGFP mice. These observations differ from the ready detection of HIF1α protein in COCs
416 matured *in vitro* at 5% oxygen. Differences in COC levels of HIF1α protein between *in*
417 *vitro* and *in vivo* matured COCs suggest a differing response of the cumulus cells to the
418 extracellular environment.

419 Nevertheless, further studies are also required to compare the expression of oxygen-
420 regulated genes in cumulus cells from COCs matured *in vitro* at low oxygen, to those
421 observed following *in vivo* maturation. In the current study, all comparisons were made to
422 cumulus cells matured at 20% oxygen. Our previous studies comparing *in vivo* matured
423 cumulus cells with those matured at 20% oxygen identified significant variations in gene

424 expression (Kind *et al*. 2013). Of note, genes previously identified by microarray as down-
425 regulated in cumulus cells matured at 20% oxygen, compared to *in vivo* maturation,
426 included *Slc2a1*, *Bnip3* and *Ndrg1* (Kind *et al*. 2013); genes identified in the current study
427 as increased at 2% and 5% oxygen when compared to 20% oxygen. Oxygen levels of 2%
428 would be considered in the lower range, based on follicular oxygen concentrations reported
429 or predicted by mathematical modelling in other species (Redding *et al*. 2008). However,
430 increased expression of HIF-regulated genes in cumulus cells at 5% oxygen is of interest.
431 Studies suggest that 5% oxygen during IVM of bovine oocytes is associated with increased
432 expression of metabolic genes in cumulus cells (Bermejo-Alvarez *et al*. 2010), and
433 improved developmental outcomes, compared to 20% oxygen (Hashimoto *et al*. 2000;
434 Hashimoto 2009; Bermejo-Alvarez *et al*. 2010; Pereira *et al*. 2010). In our studies,
435 fertilisation, cleavage and blastocyst development did not differ following maturation of
436 murine oocytes at 2%, 5%, 10% or 20% oxygen, but 2% oxygen during IVM increased
437 total and trophectoderm cell numbers in the blastocyst and reduced the percentage of inner
438 cell mass cells (Banwell *et al*. 2007). Furthermore, oocyte maturation at 5% oxygen was
439 associated with reduced fetal weight, when compared to 20% oxygen, and reduced
440 placental weight, when compared to *in vivo* oocyte maturation (Banwell *et al*. 2007),
441 whereas no differences in fetal outcomes were observed following IVM at 2% oxygen. The
442 current study identifies cumulus cell gene expression as one mechanism through which
443 oxygen concentration may influence longer-term effects; however, further studies are
444 required to compare gene expression profiles at differing oxygen concentrations to those
445 observed following *in vivo* maturation. In addition, other factors in the *in vitro*
446 environment, such as levels of glucose (Hashimoto *et al*. 2000) and EGF (Pries *et al*. 2007),
447 influence the effects of oxygen on oocyte maturation and metabolism, suggesting that the

potential for interactive effects of oxygen and media composition on developmental outcomes and cumulus cell gene expression should also be considered.

In conclusion, the current study has demonstrated significant differences in gene expression profiles within cumulus cells matured *in vitro* under varying oxygen concentrations. In particular, increased expression of genes known to be regulated by the HIF transcription factors was identified at both 2% and 5% oxygen. These results suggest that cumulus cell metabolism and function should be considered when determining optimal oxygen concentration for use during *in vitro* oocyte maturation. In addition, these results implicate HIF mediated gene expression within cumulus cells as a potential mechanism through which oxygen concentration during *in vitro* maturation could influence oocyte developmental competence.

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636

637

638 Table I. Primer sequences used for real time RT-PCR.

Gene	Genbank Accession	Primer sequence	Amplicon Size (bp)
Bnip3	BC046603	Forward - GGTTTTCCTTCCATCTCTGTTACTG Reverse – TCAGACGCCTTCCAATGTAGATC	74
Eno1	BC003891	Forward - CAAAGTGAACCAGATCGGCTC Reverse – TCCTCAGTTTCCCCAGATCG	108
Ldha	NM_010699	Forward – GGACAGTGCCTACGAGGTGATC Reverse – GCACCCGCCTAAGGTTCTTC	107
Pgk1	BC083355	Forward - TTGATGAGAATGCCAAGACTGG Reverse – AACAATCTGCTTAGCTCGACCC	131
Slc2a1	M23384	Forward – CCAGCTGGGAATCGTCGTT Reverse – CAAGTCTGCATTGCCCATGAT	76
Ndrg1	BC015282	Forward – AGTACTTTGTGCAGGGCATGG Reverse – AGGGATGTGACACTGGAGCC	94
HIF1α	NM_010431	Forward – TCAGAGGAAGCGAAAAATGGA Reverse – AGTCACCTGGTTGCTGCAATAAT	77
Acss2	BC051432	Forward - AAAAGATTGGCCCCATTGC Reverse – AATCTTCCGGAGAACTCGCC	101
Elovl6	BC051041	Forward - AGCAGTTCAACGAGAACGAAGC Reverse – CCGACCACCAAAGATAAAGGC	101
Inpp5e	AF226683	Forward - TCTGGAGATGGGAAGGTAGCA Reverse – CTCATCAAACCGGGTAGTGACA	135
Mecr	BC003864	Forward – CCCGAGACAAAAACCATCTTCA Reverse – CCGGAGCAGCTCTGTAGAACTC	90
Scd1	AF509567	Forward - CGCATCTCTATGGATATCGCC Reverse – GTGGTGGTAGTTGTGGAAGCC	104
Mrpl19	NM_026490	Forward - TTCCCGAGTACAGCACCTTTGAC Reverse – CACGGCTTTGGCTTCATTTTAAC	106
18S rRNA	AF176811	Forward – AGAAACGGCTACCACATCCAA Reverse – CCTGTATTGTTATTTTTCGTCACTACCT	91

639

Figure legends.

Figure 1. Expression of (a) *Slc2a1*, (b) *Ldha*, (c) *Eno1*, (d) *Pgk1*, (e) *Bnip3*, (f) *Ndrg1*, (g) *Hif1α* in cumulus cells following *in vitro* maturation for 17 h at 2%, 5% or 20% oxygen. All results are expressed as a fold change compared to the 20% oxygen group. Results are means ± SEM from 5 replicate pools for each oxygen treatment. Means with different superscripts are significantly different ($P < 0.05$).

Figure 2. Expression of (a) *Elovl6*, (b) *Scd1*, (c) *Mecr*, (d) *Inpp5e*, and (e) *Acss2* in cumulus cells following *in vitro* maturation for 17 h at 2%, 5% or 20% oxygen. All results are expressed as a fold change compared to the 20% oxygen group. Results are means ± SEM from 5 replicate pools for each oxygen treatment. Means with different superscripts are significantly different ($P < 0.05$).

Figure 3. Western blot analysis of HIF1α in (a) separated oocytes and cumulus cells from COCs matured for 17 h at 5% oxygen and treated for 4 h with 250 μm cobalt chloride (13-17 h of IVM); (b) COCs matured for 17 h at 2%, 5% and 20% oxygen; (c) immature COCs collected at the oocyte germinal vesicle stage (GV), COCs matured for 17 h at 5% oxygen (IVM), COCs matured *in vivo* and flushed from the reproductive tract 17 h post-hCG injection (IVV). Mouse mammary tumour tissue was included in (a) as a positive control for detection of HIF1α. β-actin levels were measured in both gels as a loading control. Experiments illustrated in a and b were replicated in three separate experiments, and results illustrated in c were replicated in two separate experiments.

Figure 4. Cumulus-oocyte complexes from C57BL/6-Tg(HRE(4)-SV40-EGFP) positive (a-c) or negative (d) mice following maturation (a) *in vitro* at 2% oxygen, (b) *in vitro* at 20%

oxygen (c) *in vivo* or (d) *in vitro* at 2% oxygen. Fluorescent images and phase contrast images are shown for each of a-d, green fluorescence in (a) represents EGFP.

Figure 1

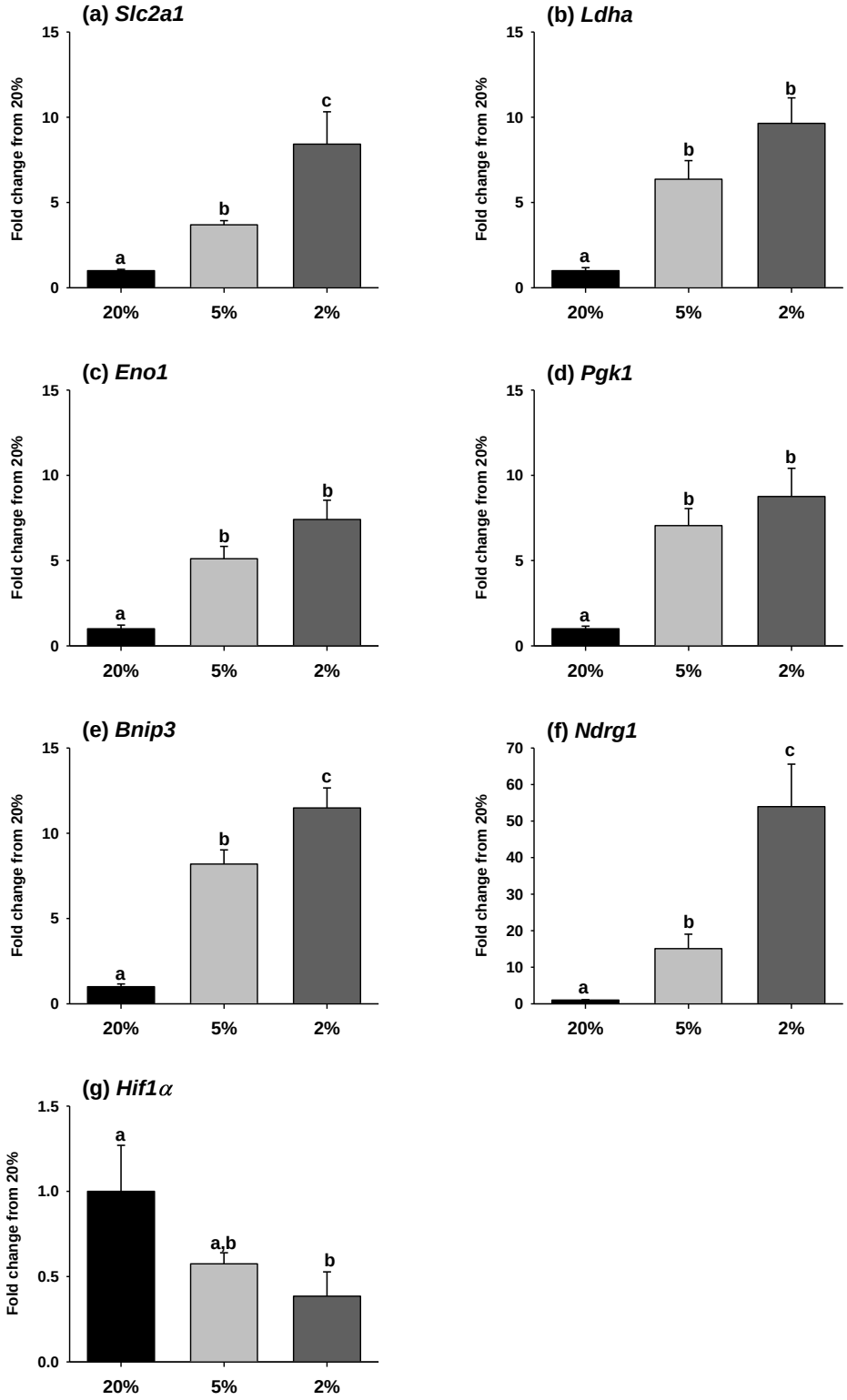

(a) Slc2a1
(b) Ldha
(c) Eno1
(d) Pgk1
(e) Bnip3
(f) Ndrg1
(g) Hif1α
Fold change from 20%
20%
5%
2%
a
b
c
a,b

Figure 2

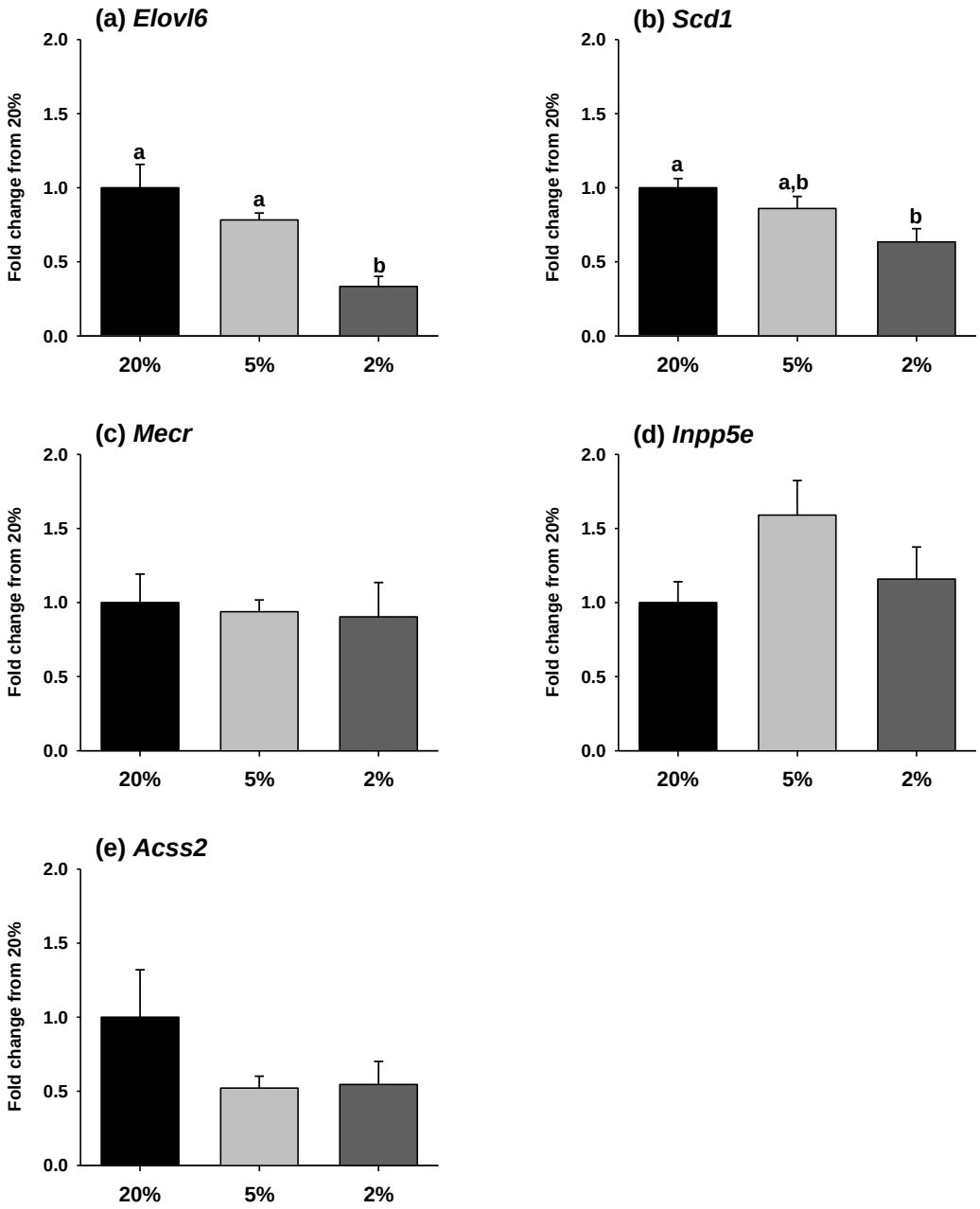

(a) Elovl6
Fold change from 20%
2.0
1.5
1.0
0.5
0.0
a
a
b
20%
5%
2%
(b) Scd1
Fold change from 20%
2.0
1.5
1.0
0.5
0.0
a
a,b
b
20%
5%
2%
(c) Mecr
Fold change from 20%
2.0
1.5
1.0
0.5
0.0
20%
5%
2%
(d) Inpp5e
Fold change from 20%
2.0
1.5
1.0
0.5
0.0
20%
5%
2%
(e) Acss2
Fold change from 20%
2.0
1.5
1.0
0.5
0.0
20%
5%
2%

Figure 3

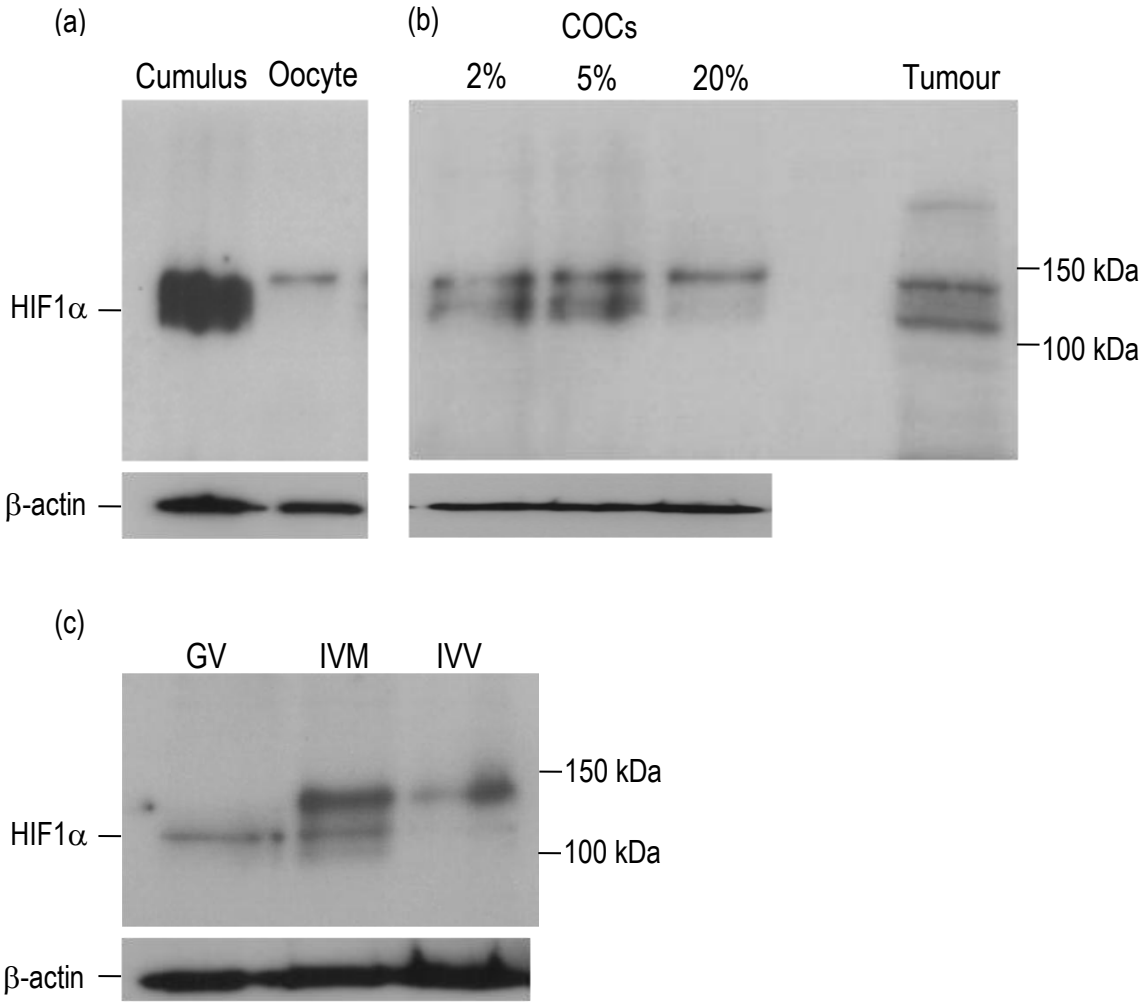
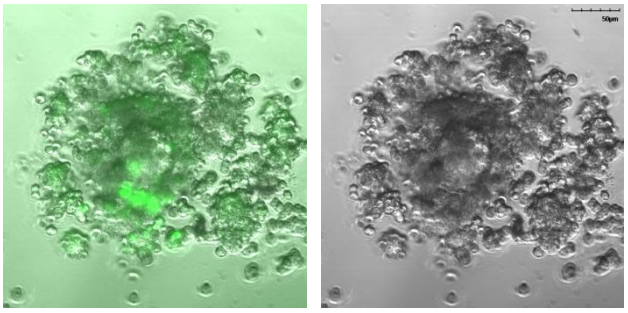
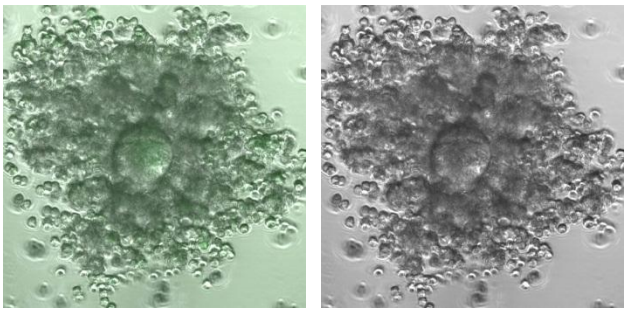

(a)
Cumulus
Oocyte
HIF1α
β-actin
(b)
COCs
2%
5%
20%
Tumour
150 kDa
100 kDa
(c)
GV
IVM
IVV
HIF1α
β-actin
150 kDa
100 kDa

Figure 4

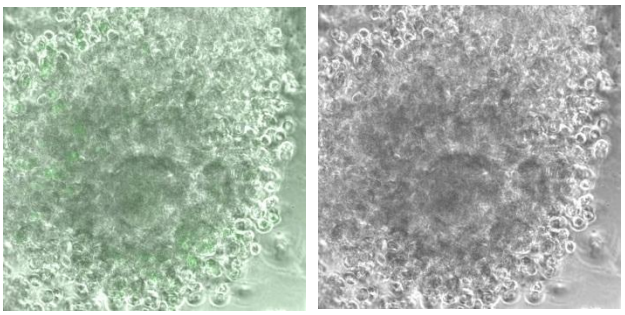
(a) 2% oxygen



(b) 20% oxygen



(c) In vivo maturation



(d) Negative control, 2% oxygen