HYPOXIA AND REPRODUCTIVE HEALTH

Hypoxia and ovarian function: follicle development, ovulation, oocyte maturationMegan Lim^{1,2,3}, Jeremy G Thompson^{1,2,3} and Kylie R Dunning^{1,2,3}

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Abstract

The ovarian follicle provides the oocyte with the ideal environment for growth and development in preparation for ovulation and fertilisation. The follicle undergoes many structural changes as it grows, including changes in vasculature, cell proliferation and differentiation and the formation of a fluid-filled antrum. These changes collectively create a low oxygen environment within the follicle. Thus, the oocyte itself develops in a potentially hypoxic environment. The survival of hypoxic tissues is controlled by hypoxia-inducible factors (HIFs) that are activated in a low oxygen state. The understanding of HIF pathways is growing across all fields of biology, and its role in ovarian development is steadily gaining clarity. One of the genes upregulated by HIF is a vascular endothelial growth factor, the main inducer of angiogenesis which is required for follicle development and corpus formation. Ovulation is also intrinsically linked to HIF activity through the ovulatory luteinising hormone surge increasing HIF expression. The role for HIF in oocyte maturation is less understood, as efforts to replicate the low oxygen environment of the *in vivo* follicle are not achievable by culturing in low oxygen alone. There is potential for other factors present *in vivo*, but lost *in vitro*, to be involved in oxygen regulation. One factor of interest is haemoglobin, the oxygen-binding protein, which brings the exciting possibility of sensitive oxygen regulation, consequently affecting HIF-regulated gene expression. A thorough understanding of oxygen regulation within the follicle would provide vital applications for the field of assisted reproductive technologies, in particular *in vitro* oocyte maturation.

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Relevance of hypoxia in ovarian biology

Oxygen is required for the survival of all living things and its deficiency results in the disruption of cellular function. However, many locations within the body exist and function in a physiologically low oxygen state (Ortiz-Prado *et al.* 2019). It is now understood that hypoxia (<6% oxygen) within organs and tissues increases the level of hypoxia-inducible factors (HIFs) (Wang *et al.* 1995, Stroka *et al.* 2001). These transcription factors function as oxygen sensors in the body, governing downstream gene expression and numerous cell processes. The first discoveries in HIFs were made by pioneering researchers in the field of medicine, culminating in the award of the 2019 Nobel Prize in Physiology/Medicine. Their research described the activation of the human erythropoietin gene in response to hypoxia (Semenza & Wang 1992) leading to developments in the treatment of anaemia (Schodel & Ratcliffe 2019) and cancers such as Von Hippel–Lindau syndrome (Kaelin 2017).

Around the same time as HIF was discovered, research in the field of reproductive biology revealed the oxygen tension in the follicular fluid of the human ovary decreased as folliculogenesis progressed (Fischer *et al.* 1992). The ovarian follicle is thought to be adapted to function in low oxygen, with the developing oocyte lying within an entirely avascular environment. The important processes of folliculogenesis, oocyte maturation and ovulation occur in response to hormones such luteinising hormone (LH), follicle-stimulating hormone (FSH), oestradiol and progesterone. There is a clear correlation between low oxygen and hormonal stimulation (van den Driesche *et al.* 2008, Tam *et al.* 2010) inducing HIF activity in mouse cumulus-oocyte complexes (COCs), leading to changes in downstream gene expression in the COC and resulting embryo (Table 1) (Kind *et al.* 2005, 2015). Importantly, hypoxia also activates the vascular endothelial growth factor (VEGF) gene which is a prime regulator of angiogenesis

Table 1 Genes expressed upon exposure to hypoxic conditions (low oxygen) and their potential roles in mouse oocyte maturation.

Cell types	Hypoxia treatment	Genes	Potential roles	References
Mouse cumulus cells	2% or 5% oxygen	<i>Slc2a1, Ldha, Eno1, Pkg1, Ndrp1, Bnip3, Elov6, Scd1</i>	Glucose uptake and glycolysis Mitochondrial function Stress protection Lipid biosynthesis Lipid biosynthesis	Kind et al. (2015)
Mouse primordial germ cell-like cells	5% oxygen	<i>Foxo3</i>	Oocyte dormancy	Shimamoto et al. (2019)

Bnip3, BCL2/adenovirus E1B interacting protein 3; *Elov6*, elongation of longchain fatty acids family member 6; *Eno1*, BCL2/adenovirus E1B interacting protein 3; *Ldha*, lactate dehydrogenase A; *Foxo3*, forkhead box O3; *Ndrp1*, N-myc downstream regulated gene 1; *Pkg1*, phosphoglycerate kinase 1; *Scd1*, stearoyl-CoA desaturase 1; *Slc2a1*, solute carrier family 2 (facilitated glucose transporter), member 1.

([Liu et al. 1995](#), [Forsythe et al. 1996](#)). Angiogenesis is a key process of ovarian function, particularly during the formation of the corpus luteum (CL) following ovulation ([Reynolds & Redmer 1999](#), [Berisha et al. 2000](#)). Further, at the time of CL formation, additional HIF-regulated genes are induced that are necessary for its development in a variety of species ([Table 2](#)) ([Meidan et al. 2013](#), [Nishimura & Okuda 2020](#)).

Much research is now focused on understanding the state of hypoxia in the ovary and applying this to clinical practices, where the implementation of a physiologically low oxygen concentration is beneficial for reproductive success for both mouse and human oocytes ([Banwell et al. 2007](#), [Gardner 2016](#)). *In vitro* follicle culture and oocyte maturation (IVM) practices are increasingly common and provide the opportunity for fertility treatments in women undergoing chemotherapy or experiencing ovarian hyperstimulation syndrome ([Brinsden et al. 1995](#), [Das et al. 2012](#)). However, during these procedures, the follicle or oocyte is removed from its *in vivo* environment and exposed to external environments with vastly different oxygen levels. The rate of successful pregnancies from IVM ranges from 7 to 40%, lower than conventional *in vitro* fertilisation ([Vitek et al. 2013](#)). The relationship between hypoxia and ovarian follicle development is evident, however, this research has yet to be translated to a clinical setting which could bring tangible improvements in IVM success.

A case for low oxygen in the ovary

Ovarian follicles undergo unique stage-wise structural changes, defining preantral from antral follicles ([Fig. 1](#)). At birth, human ovaries have a pool of primordial follicles which each contain the oocyte surrounded by granulosa cells. Upon activation of a primordial follicle into a primary follicle, a zona pellucida is formed around the oocyte and basement membrane around the surrounding granulosa cells. As the granulosa cells multiply and form multiple layers around the oocyte, the secondary follicle begins to develop. Differentiation of interstitial stroma results in the formation of a theca cell layer on the outside of the basement membrane. These structural changes define the preantral stage follicles. At this stage, FSH and LH are recognised by receptors on the granulosa and theca cells respectively, initiating the formation of a fluid-filled space among the granulosa cells termed the antrum. The antrum expands and separates the granulosa cells from the oocyte. The granulosa cells lining the follicle wall differentiate into mural granulosa cells and those adjacent to the oocyte differentiate into cumulus cells, forming a COC. A dominant follicle is selected for ovulation and non-selected follicles undergo atresia ([Fig. 1](#)). As the COC is separated from granulosa cells by the antrum, and from theca cells and vasculature by the basement membrane, this creates an avascular environment within the antral follicle ([Hirshfield 1991](#)). Aside from low oxygen due

Table 2 Genes expressed upon exposure to hypoxic conditions (low oxygen or hypoxia mimetic) and their potential roles in ovulation and folliculogenesis in various animal models.

Cell types	Hypoxia treatment	Genes	Potential roles	References
Rat luteinised granulosa cells	CoCl ₂ *	<i>Vegfa</i>	Angiogenesis, cell proliferation and differentiation	Alam et al. (2009)
Mouse primary granulosa cells	CoCl ₂	<i>Vegfa, Adamts1, Edn2, Cxcr4</i>	Ovulation	Kim et al. (2009)
Bovine granulosa cells	1% oxygen	<i>VEGF, EDN2, PTGS2</i>	Angiogenesis, cell proliferation and differentiation	Klipper et al. (2010)
	3% oxygen	<i>BNIP3, SLC2A1</i>	Luteal formation Progesterone synthesis	Nishimura et al. (2018) Nishimura et al. (2017)
Porcine granulosa cells	1% and 5% oxygen	<i>STC1</i>	Follicle growth	Basini et al. (2010)

*CoCl₂, cobalt chloride is a hypoxia mimetic.

Adamts1, a disintegrin and metalloproteinase with thrombospondin-like motifs-1; *BNIP3*, BCL2/adenovirus E1B interacting protein 3; *Cxcr4*, C-X-C motif receptor 4; *Edn2*, endothelin-2; *PTGS2*, prostaglandin-endoperoxide synthase 2; *SLC2A1*, solute carrier family 2 member 1; *STC1*, Stanniocalcin 1; *Vegfa*, vascular endothelial growth factor A.

Figure 1 Ovarian follicle development from the primordial to antral follicle stage. As the follicle transitions from preantral, gonadotropin-independent growth, to antral, gonadotropin-dependent growth, the oocyte within the preovulatory follicle is separated from the basement membrane and surrounding blood flow by a large antrum. During folliculogenesis there is an increase in theca cell layers, further separating the vasculature from the basement membrane. This together with antrum formation leads to decreasing oxygen levels within the follicle as it enlarges. Adapted with permission (Leung 2019).

to the structure of the ovary, there is also a decrease in bovine and porcine ovarian blood flow during ovulation (Wise *et al.* 1982, Magness *et al.* 1983) suggesting lower oxygen supply to the COC. Through the use of modern 3-D histological analysis, the avascularity of the inner compartment of the follicle in mice was further confirmed (Feng *et al.* 2017), providing a visual representation of the changes in the vasculature during follicular development (Fraser 2006). Therefore, a thorough understanding of the physiologically low oxygen levels experienced by ovarian cell types, across different stages of follicular development, is critical if we are to replicate this *in vitro*.

Numerous studies have endeavoured to empirically determine the oxygen levels within the ovarian follicle and understand how oxygen supply is controlled. Measurements of human ovarian follicular fluid oxygen content by aspiration indicated a decrease as follicle development progressed (Fischer *et al.* 1992). However, once the ovary is removed from the body and follicular fluid aspirated, the exposure to air results in changes in dissolved oxygen levels (Redding *et al.* 2006). Through the use of mathematical modelling, the oxygen concentration in follicular fluid was determined to be low in both human (Redding *et al.* 2008) and mouse antral follicles at the preovulatory stage (Clark *et al.* 2006). Other studies utilised oxygen sensing tools, demonstrating that large porcine follicles had lower levels of oxygen (Basini *et al.* 2004), which was also observed in bovine preovulatory, antral follicles (de Castro e Paula *et al.* 2008). This indicates that the diffusion of oxygen towards the oocyte decreases as

folliculogenesis progresses, which is supported by low oxygen consumption in cumulus cells (Clark *et al.* 2006).

Is the ovarian follicle hypoxic?

There exists a paradox in that while the maturing oocyte resides in an avascular environment, it also relies heavily on oxidative phosphorylation which requires oxygen (Thomson 1967). Thus, avascularity does not necessarily translate to hypoxia, and the follicular environment must compensate for periods that require high oxygen for metabolism. This could be facilitated by the activation of HIF target genes, which are known to be associated with angiogenesis, cell survival and glucose metabolism in other biological systems (Lee *et al.* 2004). An evaluation of HIF1A protein indicates its presence in granulosa cells of numerous animal models: mouse (Kim *et al.* 2009), rat (Zhang *et al.* 2015), bovine (Berisha *et al.* 2017), porcine (Boonyaparakob *et al.* 2005) and human (Herr *et al.* 2004, Henríquez *et al.* 2017). Furthermore, HIF1A is expressed in mature mouse oocytes and continues to be expressed after fertilisation, from the 2-cell to blastocyst stage (Takahashi *et al.* 2016).

Hypoxia-inducible factors are members of the basic helix-loop-helix (bHLH)-PAS domain family of transcription factors activated upon hypoxic stress (Semenza & Wang 1992, Wang *et al.* 1995). It consists of two subunits: HIF1A, forming part of the HIF1 protein, and HIF1B, also known as aryl hydrocarbon receptor nuclear translocator (ARNT), which bind together forming a heterodimer. The HIF1 protein is the

regulatory component that binds to hypoxia response elements (HRE) in the promoter of target genes, thereby initiating transcription. Under normoxia, HIF1A is rapidly ubiquitinated and degraded by post-translational hydroxylation (Huang *et al.* 1998). Therefore, HIF1A has a short half-life of approximately 5 min, which increases to 30 min in hypoxia as the protein is stabilised (Huang *et al.* 1998).

Due to its short half-life, studies have utilised transfection reporters to study the action of HIF in the follicle. Experiments on rat granulosa cells cultured *in vitro* show that HIF is activated by gonadotropins including FSH (Alam *et al.* 2004, 2009). The addition of human chorionic gonadotropin (hCG) to the *in vitro* culture of luteinised human granulosa cells in conjunction with hypoxic conditions (1% and 5% oxygen) lead to increased HIF1A and HIF2A expression (Herr *et al.* 2004, van den Driesche *et al.* 2008). These hormonal changes are associated with ovulation, and HIF activity also increases in ovulating and differentiating follicles in mice (Tam *et al.* 2010). This indicates HIF is intimately involved with hormonal changes in the ovary. As the preovulatory, antral follicle shows low levels of HIF activity in the mouse (Kind *et al.* 2015), it is possible that at that period of development the follicle is not hypoxic in order to support the metabolic requirements of follicle and oocyte (Thompson *et al.* 2015).

Interestingly, hypoxia is potentially involved in the maintenance of primordial follicles, which contain immature oocytes. In reconstituted ovaries derived from pluripotent stem cells, hypoxia induced oocytes to remain dormant via overexpression of Foxo3 (Shimamoto *et al.* 2019). Hypoxia also lowers the primordial follicular reserve in rats, potentially due to a reduced capacity for DNA damage and telomere repair (Aiken *et al.* 2019). The involvement of hypoxia also extends to the other stages of follicular development. There is downregulated HIF1A expression in atretic follicles compared to healthy

antral follicles in pigs (Zhang *et al.* 2018). Hypoxia also limits the growth of bovine follicles in culture through the inhibition of oestrogen receptor (Ma *et al.* 2019), whereas growth is enhanced upon increasing oxygen supply to mouse follicles (Connolly *et al.* 2019). This could potentially occur through decreasing levels of VEGF which is most required during the luteal phase after ovulation (Berisha *et al.* 2000).

The importance of HIF in ovulation

Ovarian follicle development is often likened to a tumour due to similarities in angiogenesis and levels of VEGF (Neeman *et al.* 1997). Angiogenesis, the formation of new blood vessels, is triggered by VEGF during follicle development. This leads to increased blood flow and supplies LH to the growing follicle following induction of VEGF in the theca, granulosa, and cumulus cells of the ovary (Shweiki *et al.* 1993) (Fig. 2). As angiogenesis is limited to outermost theca cells of the follicle, the avascular environment within the ovary could provide a hypoxic state which increases VEGF levels (Liu *et al.* 1995) (Fig. 2). Inhibition of VEGF is also capable of upregulating HIF in the primate follicle (Duncan *et al.* 2008). Recently, it was shown that culturing bovine granulosa cells at low oxygen upregulated genes associated with angiogenesis which are markers of early luteinisation (Baddela *et al.* 2018). Following hCG induction of ovulation in the mouse, HIF1A and HIF1B were upregulated in granulosa cells: a time when granulosa cells begin their differentiation into luteal cells (Kim *et al.* 2009). Further, when HIF1A was upregulated in hCG-stimulated luteinised human granulosa cells, there was a similar increase in VEGF gene expression (van den Driesche *et al.* 2008). Thus, it is evident that both VEGF and HIF have a strong association with one another, as evidenced by hypoxia-induced *Vegf* gene expression in numerous animal models (Table 2).

Figure 2 Association between hypoxia and vascular endothelial growth factor inducing angiogenesis in follicle and corpus luteum development. The low oxygen core of the dominant, antral follicle causes activation of hypoxia-inducible factor 1 alpha (HIF1A) protein and elevated vascular endothelial growth factor (VEGF) (Forsythe *et al.* 1996). This results in angiogenesis in the outermost cells of the follicle, increasing blood flow to the follicle. The luteinising hormone (LH) surge initiates ovulation of the cumulus-oocyte complex from the dominant follicle. After ovulation, luteinisation is characterised by continued heightened VEGF and HIF1A, resulting in angiogenesis required for corpus luteum formation (Shweiki *et al.* 1993).

Expression of *HIF1A* in response to gonadotropins is also thought to activate downstream processes of steroidogenesis and cell proliferation in granulosa cells which are critical for ovulation (Fadhillah *et al.* 2017, Baddela *et al.* 2020).

Angiogenesis is a key process in the formation of the corpus luteum, and HIF activity is maintained following its development in the mouse (Tam *et al.* 2010) and bovine (Klipper *et al.* 2010, Nishimura & Okuda 2010) (Fig. 2). Hypoxia not only signals luteal formation through inducing angiogenesis, but also luteal regression through promoting apoptosis (Nishimura & Okuda 2015). Emerging studies reveal an inverse relationship between HIF and anti-angiogenic vasohibins in the bovine follicle, indicating one of the many downstream pathways initiated by HIF in corpus luteum progression (Berisha *et al.* 2017). Therefore, a state of hypoxia is characteristic of the ovarian follicle in preparation for and during luteinisation following ovulation.

The role of HIF during oocyte maturation

The regulation of oxygen during oocyte maturation has been of great interest since before the discovery of HIFs. The process of IVM provides women with the opportunity of having a baby despite certain medical complications, such as those undergoing chemotherapy or with ovarian hyperstimulation syndrome (Brinsden *et al.* 1995, Jeruss & Woodruff 2009). However, the rate of successful pregnancies utilising IVM remains low (Cha *et al.* 2000, Child *et al.* 2001, Vitek & Robins 2013) and IVM-derived oocytes are considered of suboptimal developmental competence due to challenges in replicating the *in vivo* environment (Eppig *et al.* 1992, Gilchrist *et al.* 2011). One major difference in IVM oocytes compared to *in vivo* oocytes is altered gene expression, as seen in bovine, mouse and human studies (Tesfaye *et al.* 2009, Kind *et al.* 2013, Dorfeshan *et al.* 2018). As discussed, the *in vivo* ovarian follicle is highly complex in terms of vasculature and morphology, requiring tight regulation of hormones and oxygen, and the COC is likely in a low oxygen environment. By decreasing the oxygen concentration used during IVM of mouse oocytes, there is a marked increase in classic oxygen-regulated genes in cumulus cells of the COC together with HIF1A protein abundance (Kind *et al.* 2015). Interestingly, in the same study, COCs from *in vivo* follicles at all stages of development did not show HIF activity contrasted to those derived from IVM at low oxygen concentrations. Thus, the behaviour of HIF in the COC was different in artificial low oxygen surroundings compared to that of the *in vivo* follicle. An earlier study showed that following IVM of mouse oocytes at low oxygen, fertilisation and embryo developmental outcomes had no effect on the rate of cleavage or blastocyst rates, but increased the ratio of trophectoderm to inner cell mass cells (Banwell *et al.* 2007). The challenge then is

exploring other factors that can be altered to regulate oxygen aside from lowering the oxygen concentration.

The oxygen regulation potential of haemoglobin in the oocyte

There are a few external factors that affect oxygen regulation in the culture environment, such as glucose concentration (Hashimoto *et al.* 2000) and the presence of EGF (Preis *et al.* 2007), which when used in concurrence with low oxygen demonstrate improved oocyte competence. An unexpected factor discovered by our laboratory involves the oxygen-binding protein haemoglobin. Haemoglobin mRNA is present in high levels in mouse cumulus cells following *in vivo* maturation compared to those from COCs matured *in vitro* (Kind *et al.* 2013), as well as in human cumulus cells (Brown *et al.* 2015). Other upregulated genes included classic HIF-regulated genes which may also play a role in oocyte maturation (Table 1) (Kind *et al.* 2013). During the course of oocyte maturation *in vivo*, haemoglobin mRNA expression in mouse cumulus cells peaked at the point of ovulation, indicating hormonal regulation and at a time point associated with increased HIF activity (Brown *et al.* 2015). The localisation of haemoglobin protein within the oocyte was also altered during maturation *in vivo* indicating haemoglobin may play a role during oocyte maturation. Haemoglobin is upregulated in several non-erythroid cells that are found in areas that experience hypoxia, such as alveolar epithelial cells (Grek *et al.* 2011) and glioblastoma cells (Emara *et al.* 2014), supporting the association of haemoglobin expression with hypoxia in the maturing oocyte.

An important partner molecule of haemoglobin is 2,3-bisphosphoglycerate (2,3-BPG) which binds to the heme group of the protein and facilitates the dissociation of oxygen molecules (Benesch & Benesch 1967). The synthesis of 2,3-BPG is carried out by the enzyme bisphosphoglycerate mutase (Bpgm). In the *in vivo* matured mouse COC, *Bpgm* expression is highest during the periovulatory window, decreasing following ovulation (Brown *et al.* 2015). An increase in 2,3-BPG is commonly associated with increased demand for oxygen, such as in areas of high altitude (Lenfant *et al.* 1968). Fetal haemoglobin has a greater affinity for 2,3-BPG to allow oxygen delivery to the growing fetus from the mother (Tomita 1981). Importantly, ovarian granulosa cell tumours have upregulated *Bpgm* (Owens *et al.* 2002) which may facilitate oxygen regulation within tumour cells despite it being a hypoxic tissue. As previously stated, oxygen levels are likely to decrease in the follicle as it develops into an antral follicle, placing the oocyte in a low oxygen environment. There is a possibility of an interplay between haemoglobin and 2,3-BPG in the developing oocyte, resulting in HIF activity upon the LH surge which carries through to luteinisation (Thompson *et al.* 2015).

Figure 3 Potential role of haemoglobin in the regulation of hypoxia-inducible factors and oxygen. In the preovulatory antral follicle, prior to the luteinising hormone (LH)-surge, there are low levels of haemoglobin (Hba) and high levels of bisphosphoglycerate mutase (Bpgm). This results in low oxygen affinity to Hba, and more oxygen is released from the protein. Thus, oxygen levels are low but not hypoxic, resulting in low levels of hypoxia-inducible factor (HIF) activity. Following the LH surge, signalling ovulation, there is an increase in Hba and decrease in Bpgm, resulting in high oxygen affinity to Hba, increasing its oxygen-binding capability. This may result in a decrease in oxygen to hypoxic levels, causing high levels of HIF activity. Adapted with permission (Brown *et al.* 2015).

(Fig. 3). Haemoglobin could also play important roles in managing oxidative stress and regulating nitric oxide which is required for improved oocyte competence and embryo development (Lim *et al.* 2018, 2019).

Conclusion

The ovarian follicle is a highly specialised tissue which requires oxygen for metabolic activity during development, while structurally creating a low oxygen environment for the maturing oocyte within. It is evident that hypoxia plays an important role in facilitating oxygen regulation during follicle development and corpus luteum formation. Gonadotropic signals via FSH and LH which induce ovulation are involved in the maintenance of HIF activity, upregulating VEGF and inducing angiogenesis. Presently, the understanding of low oxygen within the follicle is beset by challenges in measuring oxygen and the differences between *in vitro* and *in vivo* matured COCs. The discovery of haemoglobin abundance within the *in vivo* human and mouse COC provides insight into one of the deficiencies present during IVM that might affect oxygen regulation and oocyte developmental competence. Successful translation of the intricacies of the *in vivo* ovarian follicle to clinical IVM might improve oocyte developmental capacity and thus provide, a better chance for reproductive success when using this assisted reproductive technology.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement

M L wrote the first draft of the manuscript. J G T provided critical feedback on the final manuscript. K R D conceived the review content, provided critical feedback and edited the manuscript. All authors edited and approved the manuscript.

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