



# **Oxygen Concentration during Oocyte Maturation in the Mouse**

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## Abstract

Follicular antral oxygen tension is thought to influence subsequent oocyte developmental competence. Despite this, *in vitro* maturation (IVM) is routinely performed in either 5 or 20% oxygen and while low oxygen has been shown to be beneficial to embryo development in many species, the effects of altering oxygen concentration during IVM have not been adequately investigated. Here we investigated the effects of a range of oxygen concentrations (2, 5, 10 & 20% oxygen) during IVM of mouse oocytes on a range of oocyte and embryonic parameters as well as fetal/placental outcome measures and cumulus cell gene expression.

While common short term measures of oocyte developmental competence such as maturation, fertilisation, and embryonic development rates were not affected over the range of oxygen levels used, more in depth investigations found several striking differences. Following IVM at 5% oxygen, the oocyte mitochondria were found to have altered patterns of both membrane potential (a measure of mitochondrial activity) and distribution suggesting altered oocyte metabolism. Following IVF, the cellular make up of embryos was investigated. In blastocysts derived from low IVM oxygen (2%) there was found to be an increased number of trophoblast cells, an increased level of apoptosis (although this was not of sufficient magnitude to account for the cell number difference) and more cells positive for both Cdx2 and Oct4 (markers of trophoblast and inner cell mass cell types respectively) suggesting a less differentiated cell type. Furthermore, following embryo transfer, the ability of the embryos to implant or develop was not altered by IVM oxygen concentration; however, fetal and placental weights were reduced in the 5% oxygen group. Cumulus cell gene expression was also examined and was found to be altered both across IVM oxygen treatment groups and when compared to cells isolated from *in vivo* derived complexes. This change in gene expression elucidates some of the many ways in which oxygen concentration during IVM may be affecting the cumulus-oocyte complex (COC) and its future development. Together, this data highlights the importance of looking past common outcome measures when determining the effects of IVM culture

conditions. The results of this study also suggest that while IVM oxygen concentration contributes to the perturbing nature of current IVM systems, it is only one of many constituents that require proper investigation, understanding and optimisation.



## **Declaration**

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Kelly M. Banwell

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## **Publications arising from these and related studies**

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# 1 Review of the Literature

## 1.1 Introduction

The ability to mature mammalian oocytes *in vitro* is both a valuable experimental model and a potentially useful clinical procedure. Currently, the sub-optimal culture systems used for maturation *in vitro* limit the use of this tool clinically and may also be leading to erroneous experimental data. Previously the area of mammalian embryo culture was in a similar predicament, but has now been revolutionised by a large body of scientific work, with resulting rates of embryo development now sufficiently high in human systems that single embryo transfer is both a viable and preferable procedure. Similar optimisation of the *in vitro* maturation (IVM) culture system could help to minimise the problems associated with IVM.

To optimise the IVM culture system, we must first understand the process of *in vivo* oocyte maturation and how the cumulus oocyte complex (COC) interacts with its environment. This understanding will allow us to more effectively mimic the *in vivo* situation. Commonly, culture media and other components of oocyte culture systems (i.e. gas constituents, temperature etc) have been adapted from somatic cell culture. This is not optimal. Due to their specialist needs, non-somatic cell types are not catered for by general cell culture systems. Also of importance, and currently not incorporated in current IVM systems, is the unique and dynamic relationship between the oocyte and its follicular environment, including the particularly intimate relationship between the oocyte and cumulus cells.

Historically, evidence suggests that adverse conditions during oocyte culture are likely to affect further development. This is the major driving factor for improvement of oocyte culture conditions. This review will endeavour to elucidate the process of oocyte development and aims to highlight the known impact of oocyte culture environments on further development as well as strategies to improve these systems.

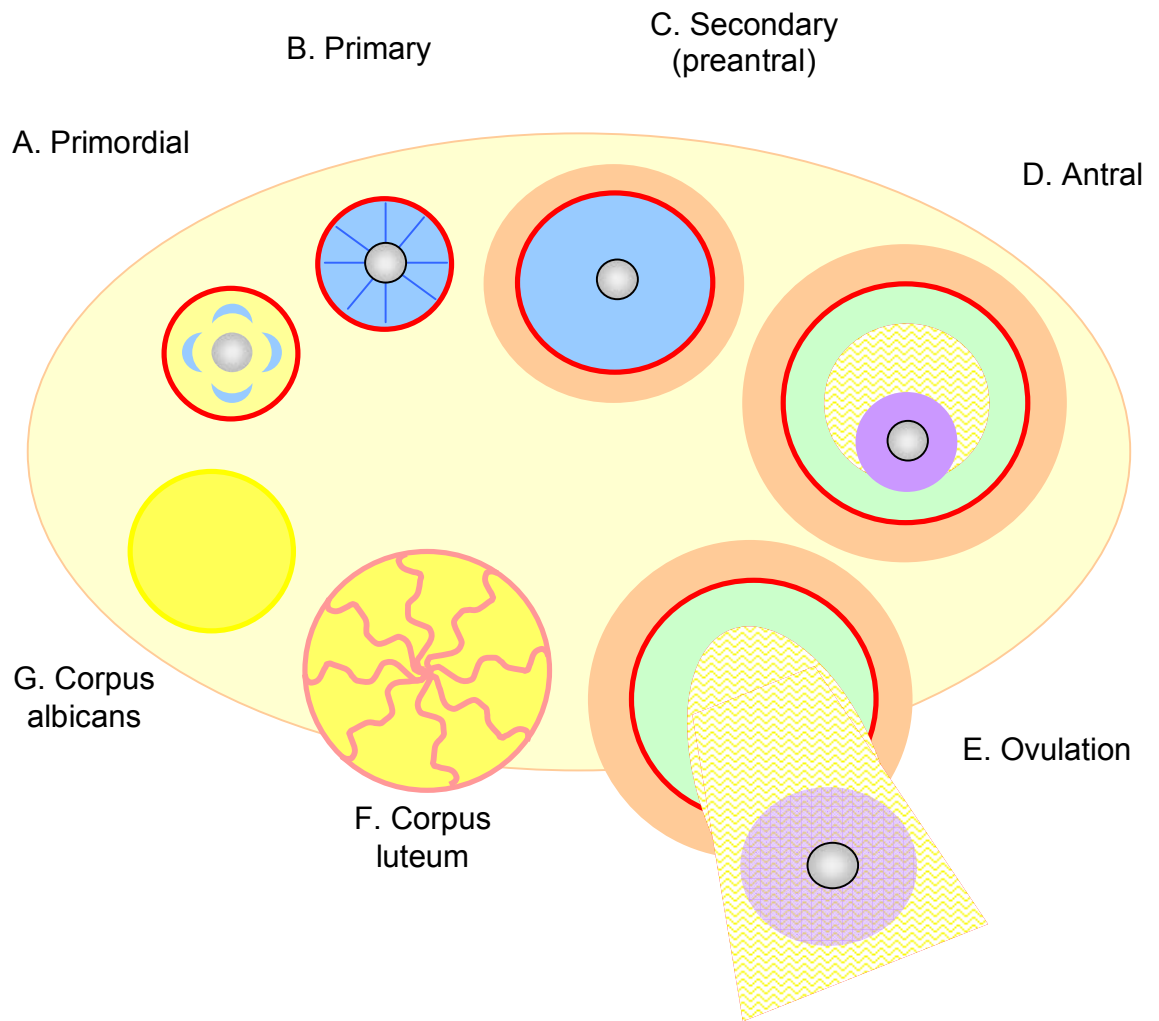
## **1.2 Oocyte development in vivo**

### ***1.2.1 Folliculogenesis***

The ovary is a dynamic organ that undergoes many changes. It contributes hormones that control the reproductive cycle, ovulation and pregnancy as well as being a store of the maternal gametes; oocytes. These oocytes are stored in unique structures known as ovarian follicles, which during the reproductive years undergo growth and development in a cyclic manner in response to the fluctuation of hormones. The culmination of each successful cycle is the ovulation of one (or more) mature COC ready for fertilisation. This complex and its surrounding follicle go through a number of stages of maturation and growth leading up to this event, the first of which occurs before birth.

The pool of primordial follicles is established during embryonic development in cows, sheep and humans and at birth in rodents and represents the total reproductive life-time supply of oocytes (Parrott and Skinner 1999). These follicles consist of a single layer of flattened pregranulosa cells enclosed in a basal lamina surrounding an inactive oocyte arrested in prophase of the first meiotic division (Figure 1.1A). The number of these follicles present in the ovary is species specific. For example the human ovary contains around 1 million primordial follicles while the bovine ovary contains approximately 150,000 (Bao and Garverick 1998, Macklon and Fauser 2001). These follicles remain in this inactive state until follicular recruitment occurs during the life of the organism.

Follicular recruitment begins as soon as the primordial follicle store has been established and it continues for the rest of life or until the ovary is depleted. There are many follicles recruited that will never ovulate. And indeed even from follicles recruited during an ovulatory cycle only one (in mono-ovulatory species) or more dominant follicle(s) will arise and be ovulated.



**Figure 1.1 A schematic of folliculogenesis in the mammalian ovary.**

(A) The primordial follicle [with an inactive oocyte (grey) surrounded by flattened pregranulosa cells (blue) and a basal lamina (red)] develops through many follicular stages once recruited; (B) the primary stage [with cuboidal granulosa layer (blue)]; (C) the secondary stage [with proliferating granulosa layers (blue) and steroidogenic theca layer (peach)]; (D) the antral stage [with follicular fluid filled cavity (yellow) and differentiated cell populations, mural granulosa cells (green) and cumulus cells (purple)]; (E) before reaching ovulation [expanded COC (light purple)]. (F) The corpus luteum develops from the remaining follicle which either maintains pregnancy or forms the regressing corpus albicans (G).

The process of recruitment is a poorly understood one. It is not known how or why particular follicles are activated from their dormant state into the cohort or why one follicle (or several) arises as dominant, but from the original oocyte pool, only about 400 follicles will ovulate in a human lifetime with a further 1000 follicles lost per month by the process of atresia (Macklon and Fauser 2001). Why other follicles do not make it to ovulation is also not known.

Once recruited, the first phase of follicular growth is the transformation of the inactive flattened pregranulosa cells to proliferating cuboidal granulosa cells. This is followed by the second phase which involves an increase in the size of the oocyte. The timing of this growth is species specific, occurring in mice when the oocyte is surrounded by approximately 10 granulosa cells in the widest cross section (Perdersen 1970, Lintern-Moore and Moore 1979). Once one full layer of granulosa cells encloses the oocyte, a primary follicle is formed (Figure 1.1B) (Picton 2001).

As the primary follicle continues to grow, the number of somatic cells present in the follicle increases rapidly (Picton 2001). The granulosa cells begin to form multiple layers and the follicle is then known as a preantral or secondary follicle (Figure 1.1C). The surrounding stroma differentiates into steroidogenic thecal cells which begin to express luteinising hormone (LH) receptors. Similarly, the granulosa cells express follicle stimulating hormone (FSH) receptors. Prior to this stage, follicle growth seems to be independent of gonadotrophin stimulation, however from this point forward follicular growth is FSH dependent (Fauser and van Heusden 1997). It is also at this stage that a fluid filled space known as the 'antrum' begins to form.

The composition of the follicular fluid (FF) found in the antrum is not completely characterised but is essentially a filtrate of venous plasma containing proteins, steroids, cytokines and growth factors as well as peptide hormones (Sutton *et al.* 2003). The granulosa cells and basal lamina restrict plasma proteins with a molecular weight greater than 250000 (Andersen *et al.* 1976). Because of the nature of this 'filtration' some of the concentrations of constituents present in FF are unchanged [globulins (Velazquez *et al.* 1977),

chloride, calcium and magnesium (Gosden *et al.* 1988)] while others are elevated [albumin and some amino acids (Velazquez *et al.* 1977; Harris *et al.* 2005), potassium (Schuetz and Anisowicz 1974)] compared to venous plasma.

In antral follicles the granulosa cells have begun to differentiate into two distinct populations, the cells associated with the oocyte known as cumulus cells and the mural granulosa cells found around the interior of the basal lamina (Figure 1.1D). The growth factor rich FF surrounds the now distinct cumulus oocyte complex. The antrum continues to swell causing the follicle to bulge from the ovary surface. The follicle wall furthest from the oocyte becomes thin due to enzyme activity. When the follicles are large, the granulosa cells acquire the ability to produce estradiol (from androstenedione derived from the theca) which elicits the gonadotrophin surge (Hansel and Convey 1983; Fortune *et al.* 2001). It is thought that perhaps this combination of increased estrogen secretion and increased responsiveness to LH/FSH determines follicular dominance (Hansel and Convey 1983; Fortune *et al.* 2001). After the gonadotrophin surge the dominant follicle ovulates, expelling the mature COC. The vasculature invades the remaining collapsed follicle from the surrounding theca to form the corpus luteum (Figure 1.1F). If the ovulated oocyte is fertilised, the corpus luteum secretes progesterone in response to hormone signals from the embryo, maintaining pregnancy. If not, the corpus luteum undergoes luteolysis, stops progesterone secretion and regresses becoming a corpus albicans. Diminished progesterone levels cause changes in the endometrium resulting in either shedding of the lining in the menstrual cycle (occurring in humans and some primates), or a degeneration in size of the lining during an oestrous cycle.

### **1.2.2 Oocyte maturation**

The acquisition of oocyte developmental competence or the ability of an oocyte to successfully develop once fertilised, occurs throughout the process of folliculogenesis and culminates in the final stage of oocyte maturation (Eppig *et al.* 1994). Final oocyte maturation can be separated into two distinct parts; nuclear maturation and cytoplasmic maturation. Nuclear maturation involves the progression of meiosis from an arrested prophase I nucleus (known as the germinal vesicle) to the metaphase II stage. The oocyte undergoes maturation in the large ovulatory follicle in response to the peri-ovulatory gonadotrophin surge before being ovulated as a mature COC, which features an expanded cumulus mass. This is necessary for ovulation, initial transport to the oviduct and for fertilisation within the oviduct (Russell and Robker 2007). By this time, the oocyte has acquired the other factors and cellular machinery required to enable subsequent development. These other factors are encompassed by the term cytoplasmic maturation and are not easily determined or defined. It is these cytoplasmic factors that represent possibly the biggest challenge to researchers attempting to understand oocyte maturation as the mechanisms are likely to be numerous and their effect on developmental competence complex.

While the oocyte is developing in this way, the follicle surrounding it is also undergoing dramatic changes, all of which have the potential to impact on the developing oocyte within. One such dramatic change occurring at this time is the rapidly expanding antrum in the follicle which is accompanied by changes in vasculature.

### **1.2.3 Ovarian vasculogenesis**

The female reproductive tract is one of the few places in the healthy adult body where the vasculature undergoes growth and degeneration on a regular basis (Reynolds *et al.* 1992; Abulafia and Sherer 2000; Hazzard and Stouffer 2000). Primordial and preantral follicles are surrounded by general stromal

vasculature and it is not until the antrum develops that the follicle gains its own vascular supply (Augustin 2000; Hazzard and Stouffer 2000). At this time the thecal layer becomes vascularised with capillary networks, but the follicle itself remains avascular with all capillaries being excluded from the basal lamina (Hazzard and Stouffer 2000). This results in the granulosa cells and oocyte remaining totally removed from the capillary network that would normally supply nutrients, substrates and hormones and remove wastes. This exclusion of vessels means that as the follicle reaches its maximum diameter before ovulation, the COC is enduring an environment seemingly void of any direct supply of oxygen and yet is able to mature and survive.

It has been long known that the ovary is one of the most well vascularised organs of the body. Although the oocyte is removed from direct vascular supply, it does perhaps have the opportunity to receive oxygen and other nutrients through diffusion across the membrana granulosa, antral fluid and cumulus cells. A large supply of oxygen and nutrients may be needed to ensure that enough passes through to oocytes and this may account for the vast vascular network.

Although the exclusion of the extensive ovarian vascular network from the follicle unit is known, the impact of this on the *in vivo* oxygen concentration of a follicle is not clearly understood. An indication of follicular oxygen levels is available for some species, due to immediate measurements of FF following follicle aspiration (Table 1.1). However, these techniques do not enable us to determine how much oxygen is available to the oocyte. Mathematical modelling of bovine and murine COCs has allowed us to calculate that across a range of oxygen levels (3 – 20%) the amount of oxygen consumed by the cumulus cells surrounding the oocyte is minimal, and thus the majority of oxygen in the FF is available to the oocyte (Clark *et al.* 2006).

There is evidence to suggest that the COC may have some control over the vascularisation of the follicle. In primate ovaries, vascular endothelial growth factor (VEGF; a hypoxia responsive gene expressed by many



tissues to stimulate angiogenesis) mRNA and protein have been localised in the preovulatory follicle to the cumulus cells surrounding the oocyte as well as the theca (Ravindranath *et al.* 1992; Kamat *et al.* 1995; Yamamoto *et al.* 1997). It is accepted that a decrease in local oxygen levels is the main reason for a tissue to stimulate angiogenesis (Hazzard and Stouffer 2000). It is unknown if this is also the cause for VEGF expression by the COC. However, it does seem that development of follicular vasculature during folliculogenesis may be of major importance to the production of a healthy follicle and subsequent oocyte.

#### **1.2.4 Follicular vascularity and developmental competence**

A preliminary study carried out in 1997 by Chui and colleagues used transvaginal Doppler ultrasonography to determine the relationship between follicular vascularity and outcome in women undergoing *in vitro* fertilisation (IVF) treatment (Chui *et al.* 1997). This study suggested that high follicular vascularity was associated with an increased pregnancy rate (Chui *et al.* 1997). Although there was no significant difference in fertilisation rates between vascularity groups (vascularity graded into 4 levels), only patients with grade 3 and 4 follicles (high vascularity) became pregnant after IVF and only those with grade 4 vascularity resulted in live births (Chui *et al.* 1997).

Gaulden proposed that the large incidence of trisomic conditions in oocytes from women > 40 years could be explained by lack of appropriate vasculature as it is known that in maturing and matured follicles, hormonal imbalances can result in imperfect microvasculature of the theca (Albertini 1992; Gaulden 1992). Gaulden also suggested preovulatory follicles that do not have appropriate vasculature, and therefore lower FF oxygen levels, cause hypoxic oocytes (Gaulden 1992). This hypoxic environment could induce a change in oocyte metabolism and pH levels resulting in chromosomal defects (Gaulden 1992). Indeed a study by Van Blerkom, Antczak & Schrader in 1997 found that human oocytes from low oxygen content follicles were associated with greater frequency of chromosomal abnormalities (Van Blerkom *et al.* 1997). In fact, of the

oocytes that exhibited chromosome displacement or abnormal alignment, a considerable 92% came from the groups developed in a follicular oxygen of <3% (Van Blerkom *et al.* 1997).

**Table 1.1 Known approximate oxygen concentration (%) levels in follicular fluid.**

<b>Species</b>	<b>Approx [O<sub>2</sub>] (%)</b>	<b>Reference</b>
Human	1.3 -5.5	(Huey <i>et al.</i> 1999), (Van Blerkom <i>et al.</i> 1997)
Porcine	7	(Knudsen <i>et al.</i> 1978)
Bovine	10 – 12	(Berg 2003)

This same study, however, also failed to demonstrate an association between dissolved oxygen content of FFs and the frequency of meiotic maturation or fertilization. However, the ability of the oocyte to develop to the six to eight cell stage post-fertilisation was reduced in oocytes from low oxygen follicles (Van Blerkom *et al.* 1997). In follicles with  $\geq 3\%$  oxygen there was a reduced frequency of chromosomal abnormalities but also a more consistent level of VEGF protein found in the FF (Van Blerkom *et al.* 1997). Not surprisingly, VEGF concentration in FF has been shown to increase both as the follicle develops (Einspanier *et al.* 2002) and to increase with maternal age (Friedman *et al.* 1997) suggesting VEGF is the mechanism by which the follicle responds to an increased need for angiogenesis. VEGF, being a hypoxia responsive gene, is induced by the hypoxia inducible factors (HIFs) in response to a low oxygen environment. When the final stages of oocyte development are mimicked *in vitro*, the addition of recombinant VEGF to bovine oocyte maturation medium improved subsequent embryo development (Einspanier *et al.* 2002). The evidence presented above indicates a role for oxygen during folliculogenesis and oocyte maturation which has potential implications for developmental competence.

The next part of this review will aim to show that when oocyte maturation is replicated *in vitro*, small changes in culture conditions to better mimic the *in vivo* environment, such as the addition of VEGF, can vastly improve developmental potential.

## **1.3 Oocyte maturation in vitro**

### **1.3.1 *In vitro* maturation (IVM)**

IVM involves the removal of oocytes and their surrounding cumulus cells from follicles before ovulation occurs, after which meiotic maturation begins either spontaneously in culture (Pincus and Enzmann 1935), or in some species, such as rat, meiosis can be maintained by cAMP modulation (Dekel 1988). IVM is a valuable experimental tool used to investigate processes occurring during cumulus expansion and oocyte maturation. Application of IVM is growing in companion animal breeding, domesticated livestock genetic

improvement and conservation of rare species as a means of gaining large numbers of oocytes for *in vitro* production (IVP) of embryos for transfer. Clinical IVM has the focus of providing an alternative source of developmentally competent oocytes without the use of significant levels of exogenous FSH administration, and these oocytes are then able to undergo routine IVF (although fertilisation is normally achieved via intracytoplasmic sperm injection) and embryo transfer procedures. Because of this, clinical IVM is most useful for women who respond inappropriately to FSH stimulation, in particular those women with polycystic ovary syndrome (PCOS). Similarly for non-PCOS patients, IVM could potentially reduce hormone use associated with clinical IVF, which has both financial and psychological benefits.

The use of IVM both experimentally and clinically faces significant challenges. Current IVM protocols result in oocytes that are less developmentally competent than their *in vivo* counterparts (Eppig and Schroeder 1989; van de Leemput *et al.* 1999; Blondin *et al.* 2002; Combelles *et al.* 2002). Nuclear maturation rates can be high (dependent on species), but nonetheless, subsequent embryo development and transfer outcomes lack the efficiency of performance in establishing viable pregnancies. For example, Holm and colleagues found bovine IVM derived zygotes have a decreased rate of development and that the cell cycle of subsequently derived zygotes was delayed (Holm *et al.* 2002). The cause and underlying mechanisms of these perturbations are yet to be fully elucidated, although they are almost certainly linked to a lack of complete cytoplasmic maturation.

The area of clinical IVM is still in its infancy, and is akin to embryo culture during the advent of IVF. Human oocyte IVM conditions are far from optimal, with a large proportion of IVM derived embryos failing to implant once transferred (Barnes *et al.* 1996; Mikkelsen *et al.* 1999). Consequently, although the process of IVM has been available, this technology has been for the most part ignored by the clinical community, especially in developed nations, where affordability of treatment is less of an impediment to access IVF treatment.

An additional and significant concern surrounding clinical use of IVM is the potential for perturbations in subsequent fetal and neonatal development that could influence long-term health. Such perturbations resulting from suboptimal embryo culture conditions and other embryo manipulations have been clearly described in animal models (Young *et al.* 1998; McEvoy *et al.* 2001; Fleming *et al.* 2004). Less clear is the role of these manipulations on subsequent developmental outcomes in the human clinical setting, as most studies report few, if any, differences in neonatal outcomes from IVF compared with natural conception data (for review see Basatemur and Sutcliffe 2008). A significant (and yet surprisingly poorly answered) question is whether IVM contributes an increased susceptibility to perturbed long-term developmental outcomes. One example of such perturbed outcomes was the mouse “Eggbert”, which was the first ever animal derived from an oocyte matured *in vitro* from a primordial follicle (Eppig and O'Brien 1996). Eggbert aged prematurely and suffered several metabolic and neurological deficiencies (Eppig and O'Brien 1998). With this in mind, the aim of the next part of this review is to describe what factors are known to affect short and long term development of oocytes.

The obvious short term consequences of sub-optimal IVM conditions are a reduced meiotic maturation competency, fertilisation and/or embryo development rate and/or decreased embryo quality. These measures are relatively easy to define and record and therefore have consequently become primary outcome measures in most IVM studies and will be discussed later in this review. However, there are other known consequences of maturation *in vitro* that are much less studied and may in fact be the mechanism by which perturbations following maturation arise.

### **1.3.2 Known alterations in gene expression and epigenetic modifications following IVM**

There are multiple levels of regulation of gene expression in the mammalian oocyte. The synthesis and storage of maternal mRNA during oocyte growth and maturation is essential for sustaining further development and can be altered or interrupted in many ways. Impairment of this process may subsequently impair embryonic genome activation (Winston *et al.* 1991; Schramm *et al.* 2003) which may be dependent on maternal genome products present in the oocyte before fertilization (Wang and Latham 1997). This impairment may result from incomplete cytoplasmic maturation caused by inappropriate IVM conditions. It has been shown that some IVM conditions (particularly the presence of industrial chemical compounds such as polychlorinated biphenyls) that affect maturation and developmental competence, also affect the extent of polyadenylation of the 3' end of mRNA (Pocar *et al.* 2001). It is also thought that the abundance or scarcity of some mRNA transcripts within *in vitro* matured oocytes may be responsible for the decreased potential of these oocytes (De Sousa *et al.* 1998). Through microarray analysis, comparing immature to mature human oocytes, many gene transcripts have been detected at higher expression levels towards maturation (Assou *et al.* 2006). In IVM oocytes this may not occur properly thus affecting further development.

Watson *et al.* (2000) found an association between maturation media and transcript abundance and development to blastocyst. Although the gene expression patterns of the maturing oocytes have been mapped (Robert *et al.* 2000; Goto *et al.* 2002; Robert *et al.* 2002; Dalbies-Tran and Mermillod 2003; Zeng and Schultz 2003), very few oocyte transcripts have been identified as potential regulators of oocyte developmental potential. De Sousa *et al.* (1998) found developmental potential of bovine oocytes to correlate with the abundance of Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\alpha$ 1. The differential expression of *in vitro* matured oocyte transcripts has not been well studied. Lonergan *et al.* (2003) found *in vitro* matured bovine oocytes

expressed significantly higher levels of transcripts for GDF9 compared to *in vivo* matured oocytes. Work in a bovine model comparing adult (competent) and prepubertal (reduced competence) oocytes showed that reduced transcript abundance of follistatin was linked to poor developmental outcomes as indicated by time of first cleavage (Patel *et al.* 2007).

Markers in the oocyte itself may not be particularly useful when it comes to predicting developmental competence because of the likely destruction of the oocyte in the process. Many groups have been looking for markers of developmental competence in the cumulus cells as small sections of these can be removed from the complex during IVM/IVF procedures. Gene expression profiles of cumulus cells can be analysed prior to embryo transfer. In studies of human cumulus obtained following gonadotrophin stimulation, PGES2, GREM1 and HAS2 gene expression have been shown to be increased in the cumulus from oocytes that form high grade embryos following fertilisation (as determined by morphological scores) (McKenzie *et al.* 2004). Similarly, microarray studies of human cumulus have uncovered a number of genes differentially expressed in the cumulus of oocytes with developmental potential (Zhang *et al.* 2005, . One such gene is pentraxin 3, a matrix protein. Pentraxin 3 is increased in the cumulus of oocytes that form morphologically normal embryos compared to those from oocytes that do not fertilise (McKenzie *et al.* 2004, Zhang *et al.* 2005). Whether these markers correlate with increased pregnancy rates has not yet been determined.

Chromatin modifications that regulate global transcription may play an important role in the progression of meiosis (De La Fuente 2006). However the mechanisms involved in the regulation of chromatin structure and how they impact transcription in the oocyte are unknown (De La Fuente 2006).

DNA methylation is the process whereby DNA methyltransferase enzymes transfer methyl groups from S-adenosyl methionine onto nucleotides, mainly cytosine, and this provides another regulatory mechanism of gene expression. DNA methylation occurs post replication and allows heritable changes in gene function



through cell divisions (reviewed by Bird 2002). The potential for ART to adversely affect the epigenetic status of the developing embryo has been widely discussed (De Rycke *et al.* 2002; Lucifero *et al.* 2004), particularly its possible role in increasing the relative risk for imprinting defect syndromes, such as Beckwith-Wiedemann and Angelman syndromes (Cox *et al.* 2002; Maher *et al.* 2003a; Maher *et al.* 2003b), but also the potential to affect fetal growth and general development. Obata & Kono (2002) demonstrated that in mice, one gene, *Impact*, is imprinted at the antral follicle stage of folliculogenesis. Similarly, several other maternal imprints (namely *Igf2r*, *Peg1* & *Peg3*) have been shown to be established late in oocyte growth (Lucifero *et al.* 2002). Incomplete methylation of maternal loci during oocyte development may have serious consequences during development and are of particular concern for IVM. Methylation status of maternal genes has not been widely studied in human oocytes and those studies in existence do not always agree (El-Maarri *et al.* 2001; Geuns *et al.* 2003). The question of whether IVM has epigenetic consequences remains unanswered although there is preliminary evidence, such as changes in methylation of the H19 locus in human oocytes (Borghol *et al.* 2006), that warrants concern.

## **1.4 Components of current IVM systems and their effects on early development**

### **1.4.1 Source of oocyte**

Short term developmental competence following IVM is significantly influenced by the source of the oocyte utilized for IVM. These factors include size of follicle and stage of oestrous cycle (Hagemann *et al.* 1999). Most fundamental is that growth of the oocyte is linked closely to developmental ability (reviewed by Trounson *et al.* 2001). As such, despite the encouraging work emerging for follicle/oocyte culture from pre-antral follicles (O'Brien *et al.* 2003), short term developmental competence following IVM is largely restricted to fully grown oocytes collected from FSH-dependent antral follicles (reviewed by Gilchrist *et al.* 2004).

### **1.4.2 Protein components**

Within large antral follicles the COC is bathed in FF until the time of ovulation. FF itself is a serum transudate modified by the thecal and granulosa cell layers which make up the follicle (Sutton *et al.* 2003). IVM media systems which closely mimic this environment have not been developed. FF contains undefined factors as well as known proteins, growth factors, steroids and metabolites, many of which are present in blood plasma (Gosden *et al.* 1988; Sutton *et al.* 2003). Not surprisingly the addition of additives such as serum and FSH to maturation media usually results in improvements in maturation and embryo development rates (Vanderhyden and Armstrong 1989; Merriman *et al.* 1998; Singh *et al.* 2003; Ye *et al.* 2005). However, the use of serum in culture systems is considered undesirable due to the unknown nature of its contents and the potential for variability (Sutton *et al.* 2003). There are alternatives to serum that are more defined such as bovine serum albumin (BSA), polyvinyl alcohol (PVA) and polyvinyl pyrrolidone (PVP). These have all been used as an alternative to serum for bovine IVM, and when used in conjunction with other factors such as hormones, can deliver comparably efficient systems (Eckert and Niemann 1995; Avery *et al.* 1998; Fukui *et al.* 2000; Mizushima and Fukui 2001). FF itself has been added to maturation media and can inhibit spontaneous maturation in high concentrations, which is thought to increase the potential for cytoplasmic maturation to occur (Lonergan *et al.* 1994; Kim *et al.* 1996; Takagi *et al.* 1998). Nevertheless, in most species, the addition of FF is not essential for IVM.

### **1.4.3 Gonadotrophins**

Exposure to hormones may not just come during the IVM culture. Gonadotrophin treatment prior to follicle aspiration is an option for improving developmental competence (Suikkari and Soderstrom-Anttila 2007). In particular, antral follicle “priming” by short-term administration of FSH appears most effective if followed by a period of FSH withdrawal (Sirard *et al.* 1999), which is suggested to initiate early atretic events, a cascade thought to mimic early cellular events associated with ovulation (Sirard *et al.* 1999). In human IVM there is

debate about the usefulness of a single large bolus of human chorionic gonadotrophin (hCG) administered prior to collection, with some studies showing a benefit for maturation timing (Chian *et al.* 2000) and a higher pregnancy rate in PCOS patients (Child *et al.* 2001), while others show no additional benefit (Chian *et al.* 2000; Mikkelsen and Lindenberg 2001; Lin *et al.* 2003; Soderstrom-Anttila *et al.* 2006).

The presence of gonadotrophins during IVM, especially FSH, is routine. Significantly, FSH promotes the mucification of the matrix by stimulation of hyaluronic acid production (Buccione *et al.* 1990). FSH has roles in cAMP regulation, Protein Kinase B (PKB) and MAPK signalling within the cumulus cells, and these are believed to assist oocyte development by assisting in the processes surrounding both meiotic and cytoplasmic maturation of the oocyte (Sutton *et al.* 2003).

#### **1.4.4 Growth factors/cytokines**

The process of follicular growth involves interaction between extra-ovarian factors such as gonadotrophins and metabolic factors, growth factors such as insulin-like growth factor 1 (IGF-1) and its binding proteins, members of the TGF $\beta$  superfamily produced locally, as well as circulating peptide hormones (Webb *et al.* 2004). Such complexity is likely to be difficult to recreate *in vitro* and may not yield beneficial effects, as the target granulosa cells are generally not present during IVM, and the cumulus cells, [which are phenotypically different from mural granulosa cells (Gilchrist *et al.* 2004)] are mostly regulated by oocyte secreted factors (OSF, see below). An example of this is the use of multiple growth factor supplements (such as insulin and IGFs) in combination with gonadotrophins during IVM, where recently it was shown that such a synthetic serum substitute actually reduced developmental competence of bovine oocytes following IVM compared with just FSH alone (Chung *et al.* 2007). Nevertheless, IGF-1 together with epidermal growth factor (EGF) have been found to stimulate short-term competence outcomes in oocytes of species such as pig and cow (Lorenzo *et al.* 1994; Grupen *et al.* 1997). *In vivo*, EGF-like peptides released by the mural granulosa cells in response to the LH surge, override the inhibitory environment of the follicle to induce

meiotic maturation at ovulation (Park *et al.* 2004). In my opinion, such peptides are underutilised in IVM systems in comparison to FSH. More work should be conducted, especially for EGF and related peptides, as they show promise with increases in maturation rate and monospermic fertilisation in IVM systems (Illera *et al.* 1998; Marques *et al.* 2007).

#### **1.4.5 Oocyte secreted factors**

During oocyte maturation, the normally compact layers of cumulus cells are separated by the production of a hyaluronic acid rich matrix to form an expanded mass. Because of the relationship between this and oocyte maturation, formation of an expanded cumulus mass is often used as a morphological indicator of oocyte maturation. Recently, it has been revealed that the processes involved in cumulus expansion (at least in the mouse), and the maintenance of cumulus cell functions are reliant on OSFs (Buccione *et al.* 1990; Dragovic *et al.* 2005). As examples, the expression of genes controlling mucification of the matrix is OSF-regulated (Dragovic *et al.* 2005), as well as the uptake of hexoses and amino acids (Eppig *et al.* 2005; Sugiura *et al.* 2005) and the production of cholesterol (Su *et al.* 2008) by the cumulus cells. OSFs are critical in maintaining the cumulus cell phenotype, which in turn, is believed to aid the process of oocyte maturation by establishing the correct environment for this to occur (Gilchrist *et al.* 2004). Studies aimed at characterising the OSFs responsible for these effects have identified a group of unique oocyte peptides that belong to the TGF $\beta$  superfamily, growth differentiation factor-9 (GDF9) and bone morphogenic protein 15 (BMP15) (also known as GDF9B). These peptides have been found to mimic the actions of an oocyte on both granulosa and cumulus cells *in vitro* (Gilchrist *et al.* 2004). Their addition to IVM media, whether in native or recombinant form, has been shown to improve blastocyst development and quality (Hussein *et al.* 2006) and improve fetal development post-transfer (Yeo *et al.* 2008). Such results reveal that IVM of COCs is likely to disturb the communication between the oocyte and cumulus leading to a disturbed microenvironment and a reduced developmental competence. Maintenance of the cumulus cell phenotype

and paracrine communication between cumulus cells and the oocyte appears critical in maximising the developmental competence of the oocyte during IVM.

#### **1.4.6 Thiol amino acids and glutathione**

The glutathione (GSH) peroxidase/reductase system is a protective mechanism activated in aerobic organisms to protect against oxidative stress (Meister and Tate 1976). Low molecular weight thiols such as cysteamine and  $\beta$ -mercaptoethanol have been used in IVM systems to induce the synthesis of GSH within the oocyte, with a concomitant decrease in levels of hydrogen peroxide (de Matos *et al.* 1996; de Matos and Furnus 2000; de Matos *et al.* 2002) and improvement in blastocyst development in several species (Gruppen *et al.* 1995; de Matos *et al.* 1996; Kito and Bavister 1997; de Matos and Furnus 2000; Gasparini *et al.* 2000; de Matos *et al.* 2002).

#### **1.4.7 Energy substrates**

The changes in energy requirements of the maturing COC have not been adequately investigated nor has the differing requirements of the oocyte and cumulus cells been properly addressed. For instance, glucose is poorly utilised by oocytes of most mammalian species examined, with pyruvate as the preferred energy substrate (Biggers *et al.* 1967; Eppig *et al.* 2000). In contrast, cumulus cells consume significant levels of glucose and are thought to provide carboxylic acids to the oocyte (Eppig *et al.* 2000). Although the oocyte does not seem to utilise much glucose directly, preferring pyruvate/lactate provided by the cumulus cells, overall glucose availability and metabolism within the whole COC is still important for oocyte maturation (Krisher and Bavister 1998; Rose-Hellekant *et al.* 1998; Sutton-McDowall *et al.* 2005). In particular, intra-oocyte levels of glycolytic activity, even if low, are correlated to developmental competence (Krisher and Bavister 1999; Spindler *et al.* 2000). Energy substrates also interact with FSH stimulation of IVM, altering the kinetics of meiotic progression (Downs and Hudson 2000; Sutton-McDowall *et al.* 2005). Maturation of

oocytes in the presence of FSH significantly increases energy substrate uptake and utilisation (Roberts *et al.* 2004; Sutton-McDowall *et al.* 2005). However, a high level (20 mM) of glucose during IVM is detrimental to subsequent embryo development (Hashimoto *et al.* 2000b). The optimal glucose concentration may differ between individual COCs as their ability to utilise it may be different and on this basis glucose utilisation by the oocyte has been suggested as a prospective marker of oocyte developmental potential (Krisher and Bavister 1999; Spindler *et al.* 2000; Sutton *et al.* 2003). Glucose alone, although sufficient, is not optimal for oocyte development with improvement following further addition of pyruvate (Downs and Hudson 2000).

Pyruvate consumption correlates with meiotic maturation with meiotically active oocytes consuming more than their arrested counterparts (Downs *et al.* 2002). This is further evidenced by the suppressive effects on pyruvate uptake upon addition of meiotic inhibitors (Downs *et al.* 2002). Amino acids can act as an energy source for the oocyte but this depends on the presence of cumulus for initial uptake (Colonna and Mangia 1983). Watson *et al.* (2000) investigated the effects of bovine oocyte maturation in a serum-free system and was able to show both improved blastocyst development rate and cell number in the presence of amino acids.

Glucosamine is an alternative hexose substrate, metabolised through the hexosamine biosynthesis pathway. The pathway is rate-limited by glutamine-fructose-6-phosphate transaminase 1 which is responsible for the conversion of fructose-6-phosphate into glucosamine-6-phosphate. Glucosamine is readily phosphorylated into glucosamine-6-phosphate, thus by-passing the rate limiting enzyme. Such upregulation of the hexosamine biosynthesis pathway is known to increase O-linked glycosylation of many intracellular signalling molecules at serine and threonines that are normally phosphorylated, including the insulin signalling pathway (Zachara and Hart 2004). Thus O-linked glycosylation of signalling molecules generally has opposing effects compared with phosphorylation and its effects on insulin signalling are inhibitory (Andreozzi *et al.* 2004; D'Alessandris *et al.* 2004). Glucosamine is utilized readily for the

production of glycosaminoglycans such as hyaluronic acid (Sutton-McDowall *et al.* 2004). So while glucosamine addition during IVM has been shown to aid cumulus expansion by diverting the use of glucose away from matrix formation (Sutton-McDowall *et al.* 2004), it was also found to severely inhibit blastocyst development (Sutton-McDowall *et al.* 2006). Furthermore, this effect was partly attributed to increased O-linked glycosylation within cumulus cells, as inhibition of glycosylation partly restored blastocyst development (Sutton-McDowall *et al.* 2006). As glucosamine is regarded as a hyperglycaemic mimetic, this may help explain why high levels of glucose during IVM are also inhibitory for subsequent development.

#### **1.4.8 Meiotic inhibitors**

It is suggested that the spontaneous maturation that occurs upon removal of the COC from the follicle occurs without the acquisition of the correct factors and machinery required to adequately support further development, with these factors being the main difference between IVM oocytes and their *in vivo* counterparts. By delaying spontaneous nuclear maturation during IVM with meiotic inhibitors, it has been proposed that continued mRNA and protein accumulation in the ooplasm should ensure complete cytoplasmic maturation and thus improve development. The most common approach is maintenance or elevation of intra-oocyte cAMP levels within the oocyte during maturation, which prevents germinal vesicle breakdown (GVBD) and meiotic progression (Eppig 1989; Conti *et al.* 1998; Conti *et al.* 2002; Mehlmann 2005). The pathway by which the level of cAMP regulates meiotic progression is poorly understood, except that it culminates in the activation or inactivation of a protein complex known as maturation (sometimes meiosis or mitosis) promoting factor (MPF) (Mehlmann 2005).

Levels of cAMP can be manipulated by the addition of a number of compounds to IVM media. Addition of cAMP analogues such as dibutyryl-cAMP (Funahashi *et al.* 1997) and activators of adenylate cyclase (Luciano *et al.* 1999; Luciano *et al.* 2004) to IVM media has been successful in delaying meiotic progression, with both showing increased cleavage and development to blastocyst stage.

Purines such as hypoxanthine, adenosine and guanosine that occur naturally in FF have been used as inhibitors of nuclear maturation (Kadam and Koide 1990). Purines are thought to act through the retention of cAMP which in turn maintains meiotic arrest (Eppig and Downs 1984). The use of purines to 'synchronise' maturation of oocytes has been well documented, particularly in porcine systems, with several studies showing an improvement in subsequent development (Funahashi *et al.* 1997; Somfai *et al.* 2003).

Phosphodiesterase (PDE) isoenzyme inhibitors have also been used to inhibit meiotic maturation due to their ability to prevent the metabolism of intracellular cAMP, thus promoting its accumulation (Thomas *et al.* 2002). As with most cAMP elevating agents, the inhibitory effect of PDE inhibitors is only transient in ungulate species (Thomas *et al.* 2002, 2004a), however, this is sufficient to positively affect development (Funahashi *et al.* 1997; Luciano *et al.* 1999; Thomas *et al.* 2004b; Bagg *et al.* 2006). In the follicle, the type 3 and 4 isoenzymes are compartmentalized to the germ and somatic cells respectively (Tsafirri *et al.* 1996; Conti *et al.* 2002; Thomas *et al.* 2002). PDE inhibitors are iso-enzyme specific for either type 3 (milrinone) or 4 (rolipram) PDEs, resulting in differential regulation of oocyte maturation by PDE inhibitors because of the accumulation of cAMP in either oocyte or cumulus cells (Thomas *et al.* 2002). In addition to increasing the accumulation of cAMP, PDE inhibitors have also been shown to maintain and/or upregulate the cell-to-cell gap junction communication between the oocyte and cumulus cells which is speculated as being partly responsible for maturation delay and improved cytoplasmic maturation (Thomas *et al.* 2004a).

Maturation can also be prevented by agents that inhibit protein synthesis or phosphorylation [e.g. cycloheximide and 6-dimethylaminopurine (Lonergan *et al.* 1997)] and some that act on cyclin-dependent kinases (Le Beux *et al.* 2003) [e.g. butyrolactone (Lonergan *et al.* 2000; Hashimoto *et al.* 2002; Wu *et al.* 2002) and roscovitine (Mermillod *et al.* 2000; Marchal *et al.* 2001; Ponderato *et al.* 2002)]. However, with the exception of butyrolactone in bovine IVM when used under low oxygen (Hashimoto *et al.* 2002), most



studies investigating transient addition of such compounds to IVM media have shown adverse effects on oocyte developmental competence.

#### **1.4.9 FF-MAS**

Follicular fluid meiosis activating sterol (FF-MAS) is a FF cholesterol derivative that has been demonstrated to mediate FSH-induced meiosis resumption in mouse oocytes in which meiosis is inhibited *in vitro* by purines, such as hypoxanthine (Byskov *et al.* 2002). FF-MAS is proposed as a signalling molecule within pre-ovulatory follicles that re-initiates meiosis within the oocyte following the LH surge (Grondahl *et al.* 2003).

FF-MAS has been evaluated for effects during IVM in several species, including mouse, pig and human (reviewed by Grondahl 2008). In many of these studies, there seems little benefit to oocyte developmental competence from the addition of FF-MAS. An exception appears to be the reduction in premature chromatin separation in oocytes from aged mice (Cukurcam *et al.* 2007). In addition, FF-MAS analogues during mouse oocyte IVM were found to significantly improve both meiotic maturation and embryo development (Marin Bivens *et al.* 2004). Nevertheless, the evidence for the benefits of the application of FF-MAS in clinical or agricultural use is lacking and more work is required to understand its value.

#### **1.4.10 Oxygen**

Early investigations into the effect of oxygen concentration during IVM both in the mouse and hamster concluded that 5% oxygen was optimal for nuclear maturation (Haidri *et al.* 1971; Gwatkin and Haidri 1974). Since this time, studies investigating the effects of oxygen during oocyte maturation have been largely limited to concentrations of 5% or above, as below this was deemed deleterious to oocyte maturation

(Haidri *et al.* 1971; Boland *et al.* 1994). Following fertilization, blastocysts derived from mouse oocytes matured at 5% oxygen had significantly higher cell number compared to those matured at 20% regardless of whether the animals were gonadotrophin-primed (Preis *et al.* 2007). However, Eppig and Wigglesworth (Eppig and Wigglesworth 1995) observed that results obtained using a 5% oxygen environment (considered optimal for nuclear maturation) are still not equivalent to the *in vivo* rate of oocyte growth or blastocyst formation. This observation also highlights the apparent importance of cytoplasmic maturation in providing factors necessary for successful embryo development.

Oocyte nuclear maturation is dependent on the oxygen dependent process of oxidative phosphorylation (Van Blerkolm *et al.* 1995). Mitochondria convert carboxylic acids to ATP using this process, during which electrons flow from NADH or FADH<sup>2</sup> through the electron transport chain crossing the inner mitochondrial membrane. The resulting electrochemical gradient causes the flow of protons back across the membrane via the ATP synthase which drives the phosphorylation of ADP to ATP. Oxygen is the terminal electron acceptor in the electron transport chain used by eukaryotes and restriction of this vital component of energy utilisation is sure to have major metabolic consequences.

As outlined above the mitochondria are responsible for production of energy substrates and they require oxygen for this process to occur normally. Because of this, and the potential for perturbations to arise from altered metabolism, they have become an area of increasing interest in both oocyte and embryo biology. Both the activity and distribution of these organelles is thought to be important in maintaining energy homeostasis. In the zygote, following fertilization, the redistribution of mitochondria in a cluster pattern around the pronuclei of the zygote is thought to influence embryo development (Barnett *et al.* 1996). If the pattern of distribution is altered by IVM conditions this may be an indicator of stress and decreased developmental potential (Muggleton-Harris and Brown 1988; Wilding *et al.* 2001). The membrane potential of the mitochondria may be a possible marker of oocyte potential as it is an indicator of mitochondrial health

and therefore of the cell. Mitochondrial membrane potential has been shown to be higher in oocytes matured *in vivo* compared to those matured at 20% oxygen but comparable to oocytes matured at 5% oxygen (Preis *et al.* 2007). Work by Van Blerkom *et al.* (Van Blerkom *et al.* 1995) suggests mitochondrial activity affects development following fertilization, with embryos having an increased likelihood of further development when a higher ATP content is present in the oocyte.

Early studies found 5% oxygen to be optimal for nuclear maturation in some species (Gwatkin and Haidri 1974). Ever since, dogma has dictated a choice between 5 or 20% (air) oxygen mixtures. Anoxia results in a high rate of oocyte development block at the GV stage (Zeilmaker and Verhamme 1974), highlighting the dependency of oocytes on oxidative phosphorylation.

Blastocyst development rates have been shown to either improve (Park *et al.* 2005a) or remain unchanged when porcine oocytes were matured in 20% oxygen (Kikuchi *et al.* 2002). IVM at 5% oxygen (5% CO<sub>2</sub>) decreased the viability of bovine oocytes in several studies, when compared to 20% oxygen (Pinyopummintr and Bavister 1995; Hashimoto *et al.* 2000b), but this was improved when the concentration of glucose in the media was increased (Hashimoto *et al.* 2000a). Maturation of mouse oocytes at 20% oxygen results in a reduced metabolism compared to those matured under 5% oxygen as measured by glucose uptake and lactate production (Preis *et al.* 2007). Because of this confusion, the use of 5 and 20% oxygen during oocyte maturation persists in laboratories when there is no established benefit for their use. The reason such confusion exists is due to differences in experimental design, namely the choice of maturation media, type of culture system, the species used as well as the exclusion of other oxygen concentrations.

Lacking from current work on oxygen concentration during IVM is a study of the cumulus cells surrounding the oocyte. The cumulus cells are located on the periphery of the complex and thus must most certainly act

as a buffer for the oocyte. The effect of the oxygen environment during maturation on the health of the cumulus cells has not been investigated.

Also lacking from the IVM literature are studies investigating the long-term effects of IVM culture conditions, including the effects of oxygen. Developmental competence is generally measured by the ability to reach the blastocyst stage of embryo development while effects occurring post-implantation are largely neglected. The next part of the review focuses on the few studies of these effects.

## **1.5 Long term consequences following IVM**

Although morphology scores and rates of development give us some basic information about the oocyte developmental competence, we are also aware that these scores are not adequate measures of full developmental potential, which should be measured by the ability of the oocyte to form a viable pregnancy and a healthy offspring. Unfortunately, long term measures of the consequences of IVM are more difficult to measure and the experimental execution more demanding. Lower implantation rate and perturbations to the conceptus and the adult offspring are of particular concern for both clinical ART and agricultural IVP. The current reality is that little is known about the long term outcomes following IVM in most experimental models and also when applied to clinical IVM. Furthermore, these questions are unlikely to be answered quickly as children derived from IVM oocytes are not yet of adult age nor present in great enough numbers to investigate meaningfully. However, some studies have been performed in animal models and a few small human follow-up studies have now been published. The next part of this review focuses on the results of these.

Following transfer, Thompson et al (1995) showed that regardless of whether ovine embryos were IVM or *in vivo* derived this had no effect on their ability to produce pregnancies. This was despite the differing development rates and morphology of the embryos. However, IVM derived pregnancies in this study were

also associated with fetal overgrowth, also known as large offspring syndrome (LOS) (Thompson *et al.* 1995). LOS is a known consequence of *in vitro* embryo production as well as exposure to some perturbing *in vivo* environmental factors such as maternal diets high in urea, and occurs most notably in sheep and cattle (reviewed Young *et al.* 1998). Because embryo culture is a known cause of LOS it is sometimes difficult to separate the effects of embryo culture from those related to IVM. By using a system of *in vivo* culture where zygotes are transferred back into a ewe for the embryo culture period, Holm *et al.* (1996) were able to suggest that IVM does result in larger offspring. In cattle, IVM derived pregnancies were also shown to result in heavier and longer calves but this effect was dependent on duration of IVM (Park *et al.* 2005c).

There are few studies in the mouse investigating different IVM conditions and their impact on subsequent fetal development. Early studies recognized that fetal survival of IVM-derived offspring was lower than for those derived from *in vivo* matured oocytes (Schroeder and Eppig 1984). Of significance has been the recent report by Yeo *et al.* (2008) revealing IVM in the presence of recombinant oocyte-secreted factor, GDF9, improved fetal survival following transfer.

IVM has been used in human systems with successful pregnancies recorded (Trounson *et al.* 1994; Barnes *et al.* 1996; Cha and Chian 1998; Chian *et al.* 1999; Mikkelsen *et al.* 1999; Cha *et al.* 2000; Kim *et al.* 2000; Smith *et al.* 2000; Mikkelsen and Lindenberg 2001; Child *et al.* 2002). Very few studies of human obstetric and post-natal outcomes have been performed as the numbers of children born from this technology are still reasonably low. Adult health cannot be investigated at this time as there are no adults derived from IVM oocytes.

Following IVM the number of embryos transferred tends to be greater, resulting in an implantation rate per embryo that is significantly lower than for traditional IVF. However, in the pregnancies resulting from IVM few complications have been reported and both birth weight and gestational age fall within the normal range (Cha *et al.* 2005; Mikkelsen 2005). In PCOS patients, pregnancy rate following IVM appears to be slightly

lower than that following traditional IVF (Cha *et al.* 2000). However, many complications that lead to pregnancy loss are much higher in these patients and this may be related to the nature of this syndrome (Copperman *et al.* 2000; Ellenbogen *et al.* 2002). Pre-term birth rates occur at a reasonably low level (4 – 13%) when put in context of an ART pregnancy (Cha *et al.* 2005; Mikkelsen 2005; Soderstrom-Anttila *et al.* 2006; Suikkari and Soderstrom-Anttila 2007). Rate of malformation has been reported as 5.3% in PCOS patients with one documented case of soft cleft-palate (Cha *et al.* 2005; Mikkelsen 2005). Analysis of the rate of congenital defects in IVM children is currently unfeasible due to the small population. IVM children have been found to have minor developmental delays either very early in their development or during the first few years of life but overall neuropsychological development was considered in the normal range (Shu-Chi *et al.* 2006; Soderstrom-Anttila *et al.* 2006).

The impact of individual IVM culture conditions on long-term outcomes such as implantation rate, pregnancy success and fetal and adult health has not been adequately investigated in any species. The ability of an oocyte to be fertilised and form a blastocyst are not adequate measures of perturbation. It is not enough to get embryos for transfer. Work must be done to ensure the embryos used for ART procedures have the highest potential to develop normally.

## **1.6 Significance**

The early stages of development are critical in determining fetal development and longer-term adult health (reviewed by Barker 1998). The mechanisms by which the perturbation of early development affects adult disease susceptibility are not clearly understood. However, it is clear that current *in vitro* environments used during ART are inferior to that which is experienced *in vivo* and it is likely that this has some influence over early development. The environment used for IVM of oocytes is no exception.

*In vitro* embryo production is a multi-step process. Each of these steps plays a role in determining the fate of the zygote. It is likely that the period of culture post-fertilisation is most critical in determining the quality of the blastocyst (Loneragan *et al.* 2003). However, the remaining processes must also be optimised before we can expect '*in vivo* like' results.

It has been proposed that a system of human IVM be used as an additional step prior to carrying out IVF for individuals who respond inappropriately to gonadotrophin stimulation or as an alternative to stimulation for all patients. If IVM is to be used successfully either in the clinical environment (i.e. to produce healthy offspring) or as an experimental model (i.e. to produce high quality and robust data) more work is required to improve culture conditions. In my view, the *in vitro* environment will be fundamental in influencing developmental competence of the oocyte in both the short and long-term. Increased understanding of this influence will improve the efficiency, and most likely, safety of the IVM procedure.

The most successful way of improving the *in vitro* environment is to mimic the *in vivo* environment. As shown in the above review, many IVM culture constituents are being studied and improved in this way. Measures of the oxygen concentration in FF have been made and are considerably varied across species. However, regardless of species, IVM is still routinely performed at 5 or 20% oxygen. Direct measurement of mouse FF oxygen concentration is technically unfeasible and thus mouse IVM is performed under both of these concentrations without any *in vivo* precedent at all. Considering the energy production in the oocyte is reliant on oxygen, metabolism (and thus development) of the oocyte could be perturbed by inappropriate oxygen conditions leading to erroneous data and poorer outcomes. It was the aim of this study to address the deficiency of knowledge in this area.

## 1.7 Thesis aims

*In vivo*, the oxygen environment varies across the follicle population in the ovary, across species and between members of the same species. The reduced availability of oxygen in the follicle has been associated with chromosomal abnormalities and reduced pregnancy rate in humans. However, how the COC responds to the oxygen environment during maturation is unknown. Moreover, the consequences of the oxygen environment during maturation on subsequent embryonic and fetal/placental development are yet to be adequately investigated.

With these points in mind the aims of this study were:

- To investigate the effect of a range of oxygen concentrations during IVM of the oocyte on subsequent development of the pre-implantation embryo.
- To determine the effect of oxygen concentration during IVM on commonly measured parameters of developmental capacity in the embryo and oocyte.
- To determine the effect of oxygen concentration during IVM on post-transfer outcomes.
- To determine if and how gene expression by cumulus cells is altered by oxygen concentration during IVM.

The following hypotheses were proposed:

- Oxygen level during IVM affects the ability of the oocyte to mature, fertilise and/or once fertilised, develop to blastocyst stage;
- oxygen concentration during IVM alters parameters in the oocyte and embryo associated with developmental competence;
- outcomes following transfer are affected by the oxygen concentration used during IVM; and
- gene expression in the cumulus cells is altered by oxygen environment in the IVM system.





## **2 General materials & methods**

## **2.1 Chemicals and solutions**

All chemicals were purchased from Sigma Chemical Company (St Louis, USA) unless otherwise stated. All media, buffers and solutions were prepared using Milli Q water (Millipore Corporation) and stored in Nunc Brand Products (Nunc International, Roskilde, Denmark). Alpha MEM (catalogue number 12000-022) was purchased from Invitrogen (Carlsbad, USA). The following techniques were used in the generation of materials for all experiments. Specific techniques are outlined in the appropriate chapter.

## **2.2 Murine oocyte maturation**

### ***2.2.1 Ovarian stimulation procedure***

All experiments were conducted according to the National Health and Medical Research Council of Australia guidelines for the use of animals and following approval from The University of Adelaide and Institute of Medical and Veterinary Science ethics committees. For all IVM experiments, cumulus-oocyte-complexes were isolated from female hybrid CBAB6F1 mice (21 days old) that received 5 IU equine chorionic gonadotrophin (eCG) (Folligon serum gonadotropin; Intervet, Boxmeer, Holland) injected intraperitoneally 46 h prior to oocyte collection.

### ***2.2.2 Cumulus-oocyte complex collection***

COCs were isolated in HEPES-buffered  $\alpha$ MEM medium supplemented with 50  $\mu$ g/ml streptomycin sulphate, 75  $\mu$ g/ml penicillin G and 5% fetal bovine serum (FBS) (Invitrogen) by gently puncturing visible antral follicles present on the ovary surface with a 30 gauge needle. Germinal vesicle stage oocytes with an intact vestment of cumulus cells were collected and pooled from a minimum of 4 animals.

### **2.2.3 Oocyte *in vitro* maturation**

COCs were matured (10 per drop) in 100  $\mu$ l drops of bicarbonate buffered  $\alpha$ MEM supplemented with 50  $\mu$ g/ml Streptomycin, 75  $\mu$ g/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). Maturation of COCs under different oxygen concentrations was performed for 17 h at 37 C in modular incubation chambers (Billups-Rothenburg, Del Mar, USA) filled with test gas mixtures. The gas mixtures used were 2, 5, 10 or 20% oxygen (6% carbon dioxide and balance of nitrogen). Hereafter, these gas mixtures are referred to as simply 2, 5, 10 and 20% oxygen. For some experiments the 10% oxygen treatment group was not included. Culture dishes were prepared a day ahead and were allowed to equilibrate in the modular incubation chambers at 37 C overnight.

### **2.2.4 Oocyte *in vivo* maturation**

For some experiments, *in vivo* matured oocytes were generated. CBAB6F1 mice (21 days old) received a dose of 5 IU eCG and 5 IU hCG (Pregnyl; Organon) injected intraperitoneally 61 h and 13 h prior to fertilisation respectively. This ensured the *in vivo* matured oocytes were at the same developmental stage as the IVM oocytes and were receptive to fertilisation. *In vivo* matured ova were collected from the oviduct into HEPES buffered  $\alpha$ MEM medium.

## **2.3 In vitro fertilisation (IVF)**

Both cauda epididymis and ductus deferens were dissected from a CBAB6F1 male (selected by proven reproductive ability and mated at least 3 days previously) and placed in a 1 ml drop of equilibrated (5% O<sub>2</sub>, 6% CO<sub>2</sub>, balance of N<sub>2</sub>) bicarbonate buffered  $\alpha$ MEM medium supplemented with 50  $\mu$ g/ml Streptomycin,

75 µg/ml Penicillin G and 3 mg/ml fatty acid free BSA (ICPbio, Auckland, New Zealand) under oil. Sperm were liberated from the epididymis by puncturing the section closest to the ductus deferens with fine forceps. Care was taken to avoid the section furthest from the ductus deferens containing immature sperm. Remaining sperm were then squeezed down the length of the ductus deferens using fine forceps towards the cut end. Tissue debris was removed. The sperm drop was then incubated for 1 hr to allow capacitation of sperm. To an equilibrated 90 µl drop of the above medium under oil, 10 µl of capacitated sperm were added before ova were prepared. Following maturation, ova were washed twice in the above equilibrated medium before being incubated with sperm for 4 h.

## **2.4 Embryo culture (IVC)**

After incubation with the sperm for 4 h, the eggs were washed 3 times in potassium simplex optimised medium (KSOM) without amino acids but containing L-alanyl-L-glutamine (1.0 mM) (Glutamax; Invitrogen) to remove all excess sperm and cumulus cells and then placed in fresh 20 µl drops of KSOM under oil and cultured overnight. Embryos that cleaved to the two-cell stage were transferred to a fresh drop of KSOM under oil and cultured for 72 h, except when morulae were needed when culture was terminated at 48 h. Regardless of IVM conditions, all fertilisation steps and embryo culture was performed under an atmosphere of 5% O<sub>2</sub>, 6 % CO<sub>2</sub> and balance of N<sub>2</sub> at 37 C.

## **2.5 Statistical analysis of results**

Maturation status and pronuclear formation data is presented as mean and all other data are presented as mean ± standard error of the mean (SEM). All statistical analyses were carried out using either SigmaStat for Windows Version 2.03 or SPSS for Windows (SPSS Inc, Chicago, USA). A probability level of 5% (P < 0.05) was taken as significant for all analyses. All data was assessed using a one-way Analysis of Variance (ANOVA) with a Bonferroni post-hoc test with the exception of maturation status and pronucleus formation

(fertilisation), which was analysed using Chi-square analysis and cleavage and blastocyst development data, which was subjected to arcsine transformation before analysis by one-way ANOVA. Fetal and placental measures were analysed using a General Linear Model Univariate analysis with litter size as a covariate with Bonferroni post-hoc test. In addition to a one-way ANOVA, the inner cell mass numbers were Log transformed prior to analysis. A Tukeys HSD post-hoc was used for apoptosis data.

### **3 Oxygen during in vitro maturation; effects on early development**

### 3.1 Introduction

In the large pre-ovulatory follicle, where the oocyte undergoes maturation *in vivo*, the oocyte is removed from any direct oxygen supply, with the follicular vasculature being totally excluded by the basal lamina (Hazzard and Stouffer 2000; Plendl 2000; Tamanini and De Ambrogi 2004). As a result, the oocyte must gain oxygen from the surrounding vasculature by the process of diffusion across both the cellular layers of the follicle (mural granulosa cells and cumulus cells) and the fluid filled antrum. While vascularisation of the follicle increases as the follicle grows, particularly at this large antral stage, it is not directly known how much oxygen is able to diffuse across the follicle wall or how much is available to the oocyte.

In larger species, the oxygen content of FF has been measured immediately following oocyte aspiration giving us an indirect measure of the amount of oxygen able to diffuse into the FF from across the follicle wall. Interestingly, this has been shown in both bovine and humans to vary considerably between subjects and between follicles in the same subject (Van Blerkom *et al.* 1997; Huey *et al.* 1999; Berg *et al.* 2003). Some studies have attempted to correlate this oxygen content in the follicle with oocyte quality. In the human, these studies were able to show that the dissolved oxygen content of human FF ranges between 1.3% to 5.5% oxygen, but were unable to show that this oxygen content had any association with frequency of oocyte meiotic maturation or fertilisation, embryo cleavage or morphology (Van Blerkom *et al.* 1997; Huey *et al.* 1999). However, following fertilisation, the ability of oocytes from low oxygen follicles (<1.5% O<sub>2</sub>) to develop to the six to eight cell stage was found to be reduced (Van Blerkom *et al.* 1997). Furthermore, Huey *et al.* (1999) were also able to demonstrate that reduced follicular vascularisation (measured by colour Doppler analysis) was negatively correlated with day 3 cleavage and embryo morphology. This data suggests that *in vivo*, the follicular oxygen availability has some role to play in oocyte developmental competence.



*In vitro*, the optimal oxygen level for oocyte maturation has not been determined and normally standard oxygen concentrations used for embryo culture (i.e., 5% O<sub>2</sub> or air [21% O<sub>2</sub>]) have been applied during IVM. Little is understood about how the *in vivo* follicular oxygen environment during the final stages of oocyte maturation or the oxygen environment during IVM influences the subsequent developmental potential of the oocyte. Where oxygen effects have been investigated during IVM, there are conflicting results. Using bovine COCs, IVM at 5% oxygen (5% CO<sub>2</sub>, balance of N<sub>2</sub>) dramatically decreased the viability of oocytes in several studies, when compared to 20% oxygen (Pinyopummintr and Bavister 1995; Hashimoto *et al.* 2000a), whereas in contrast, oxygen levels appear to have no effect on pig oocytes (Park *et al.* 2005b). Furthermore, blastocyst development rates have been shown to both improve (Park *et al.* 2005b) or remain unchanged when porcine oocytes are matured in 20% oxygen (Kikuchi *et al.* 2002) compared to lower oxygen levels (7 and 5% oxygen respectively). Media composition may play a role in these discrepancies, as Hashimoto and colleagues (2000a) also reported that 5% oxygen during IVM supports improved bovine embryo development if the concentration of glucose is increased, possibly because of increased ATP production via increased glycolysis.

It is clear that confusion exists over the effect of oxygen concentration during IVM on further development. This confusion is largely due to the variety of species and culture systems (such as media composition) used, and partly due to study design. Concentrations other than 5 and 20% oxygen are rarely included within experimental designs. Also, most studies in this area do not use oxygen concentrations less than 5%, as early studies suggested lower levels were detrimental (Haidri *et al.* 1971; Boland *et al.* 1994). Since these early studies, our understanding of culture conditions and handling techniques have improved, which may change the results if studied now. The range of oxygen concentration measured *in vivo* includes levels below 5% (see Chapter 1; Table 1.1). Nevertheless, most common IVM culture systems use an oxygen level of 20%. We determined that a comprehensive study across the spectrum of oxygen concentrations (encompassing levels below 5% and including 20% oxygen) was needed to help define the effects of

oxygen concentration during IVM on developmental competence. The most widely used measure of developmental competence is the ability to develop to blastocyst stage. Therefore the aim of this study was to determine the effect of varying oxygen concentrations during IVM on subsequent pre-implantation development.

## **3.2 Materials and methods specific to this chapter**

Mouse ovarian stimulation, oocyte collection, IVM, IVF and IVC were performed as outlined in Chapter 2.

### **3.2.1 Maturation status assessment**

To determine if IVM oxygen concentration altered rates of oocyte nuclear maturation, the following procedure was used. IVM was conducted under 2, 5, 10 or 20% oxygen concentrations, Following IVM a sub-set of COCs from each treatment group were treated with 50 U/ml ovine hyaluronidase and all cumulus cells were removed with the aid of gentle pipetting. Denuded oocytes were collected, washed through fresh medium, followed by phosphate-buffered saline (PBS) and were then fixed in ethanol: glacial acetic acid (3:1). Oocytes were fixed for a minimum of 48 h before staining with 1% aceto-orcein solution for 30 min at RT. Orcein stain was cleared from the oocytes using a 1:1:3 solution of glycerol, acetic acid and water. Slides were sealed and nuclear structures were visualised under phase contrast microscopy using a Leica DM IRB microscope (Leica, Germany). Metaphase II (MII) was indicated by the presence of a MII spindle within the oocyte and a polar body in the perivitelline space.

### **3.2.2 Fertilisation assessment**

To assess the effect of IVM oxygen concentration on fertilisation rate, the following procedure was used. After the 4 h fertilisation and following 3 h of culture, a subset of presumptive zygotes was visually assessed for presence of the male pronucleus using orcein staining as previously described.

### **3.2.3 Differential nuclear staining**

To determine a further quality parameter of subsequent blastocysts, the number of cells within the inner cell mass (ICM) and trophectoderm (TE) of blastocysts was assessed by differential staining. Blastocysts were placed in 0.05% pronase solution at 37 C for up to 5 minutes to remove the zona pellucida before being washed in a HEPES buffered version of G1 medium (H-SG1) (Lane and Gardner 2003). This was followed by incubation with ice-cold 0.5% (w/v) 2,4,6-trinitrobenzene sulfonic acid (TNBS) solution for 10 min at 4 C in the dark followed by a wash in H-SG1 medium. Embryos were then incubated in a 0.1 mg/ml solution of anti-dinitrophenyl-BSA antibody (anti-DNP) for 10 min at 37 C. After a wash in H-SG1 medium, complement mediated lysis was induced by incubation with complement solution [guinea pig serum diluted in H-SG1 medium with 20 µg/ml propidium iodide (PI)] at 37 C for 5 min. Finally, embryos were briefly washed in H-SG1 medium, transferred to 25 µg/ml bisbenzimidazole (Hoechst 33342) in ethanol and stored overnight at 4 C in the dark. Embryos were stored in 100% alcohol in the dark at 4 C until visualised. Stained embryos were mounted in glycerol on a microscope slide and overlaid with a coverslip. The differential colour of the nuclei was examined using an Olympus AH-3 microscope (Olympus, Tokyo, Japan) whereby under ultraviolet excitation the ICM (stained with Hoechst 33342) appeared blue and the dual stained TE cells appeared pink/red. Number of ICM and TE cells was obtained for each embryo, and the ratio of ICM to total number of cells calculated.

### **3.2.4 Embryo apoptosis assessment**

To determine if different oxygen treatments during IVM (10% oxygen treatment group was not included in this analysis due to the similarity of previous results to the 5% group, see 3.3.4) had effects on degree of apoptosis in blastocysts, apoptotic DNA was detected in blastocyst stage embryos using a terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick-end labelling (TUNEL) assay (Roche Diagnostic, Penzberg, Germany) according to the manufacturer's instructions. Blastocysts derived

from *in vivo* matured oocytes were also included in this analysis to provide a baseline level of apoptosis in the mouse blastocyst. Briefly, blastocysts were washed in PBS supplemented with 3 mg/ml polyvinyl pyrrolidone (PVP) twice before being fixed overnight in 4% paraformaldehyde in PBS (pH 7.4) at 4 C. Embryos were then washed twice in PBS/PVP, permeabilised in 0.5% Triton X-100 for 1 h at room temperature and washed twice in PBS/PVP. Positive controls were then created by incubation with DNase 1 (0.005 U/ $\mu$ l) for 20 min at 37 C, which cleaves all DNA, after which these embryos were washed in PBS/PVP twice. All embryos were incubated in fluorescein-conjugated dUTP and terminal deoxynucleotide transferase (TdT) (TUNEL reagents, Roche Diagnostic) for 1 h at 37 C in the dark. Negative controls were incubated in fluorescein-dUTP in the absence of TdT. After TUNEL, embryos were washed in 0.5% Triton X-100, PBS/PVP twice and RNase buffer (8.76 mg/ml Tris, 0.58 mg/ml NaCl and 0.57 mg/ml MgCl<sub>2</sub>) and then counterstained with PI (0.05 mg/ml) plus RNase A (0.05 mg/ml) for 1 h at room temperature in the dark. Embryos were then washed twice in 0.5% Triton X-100 and once in PBS/PVP and mounted in glycerol for immediate analysis by fluorescence microscopy.

### **3.2.5 Immunohistochemistry of morulae**

To investigate if cell fate was altered in subsequent embryos due to different oxygen treatments during IVM, markers of TE and ICM cells were localised by immunohistochemistry in morulae. Both the primary and secondary antibodies used in this study are listed in Table 3.1. All antibodies were obtained from the sources indicated in Table 3.1. Normal donkey serum for blocking non-specific binding was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA).

Morulae derived from oocytes matured under different oxygen concentrations (2, 5, 20% or *in vivo*), were fixed in 4% paraformaldehyde in PBS for 1 h at room temperature and washed twice in PBS before being placed on Cell-tak (Beckton Dickinson Biotechnology)-coated large coverslips and allowed to settle for approximately 5 minutes. Care was taken to avoid drying out of the sample and additional PBS was added

to the slide with a mouth pipette if needed. After all embryos had settled satisfactorily onto the surface of the coverslip, additional PBS was removed with a mouth pipette and replaced with fresh solution.

The antibodies described in Table 3.1 were used to determine the presence of two transcription factors, Oct4 and Cdx2 (markers of ICM and TE cell lineages, respectively) in morulae following treatment during IVM (2, 5 and 20% oxygen and *in vivo* control). Embryos were neutralised in 0.1M glycine for 5 min at RT, washed 3x in PBS and then permeabilised in a 0.25% Triton-X 100 solution for 15 minutes. They were then washed a further 3x in PBS before a 5% normal donkey serum in PBS blocking solution was applied for 30 min at room temperature to minimise non-specific binding. Morulae were incubated in primary antibodies at the dilutions specified in Table 3.1 at 4 C overnight. A control sample was also generated in which the primary antibody was omitted.

Following this incubation, embryos were washed 3x in PBS before incubation with the appropriate fluorophore-conjugated secondary antibody (Table 3.1) for 2 h at room temperature. Following 3x PBS washes, nuclei were counterstained with the nuclear stain, DAPI (3  $\mu$ M) in PBS for 10 minutes at RT to aid localisation. Small coverslips were mounted onto the sample coverslip using a 1:1 mix of ProLong Gold antifade reagent (Molecular Probes, Eugene, OR) and glycerol. Morulae were imaged immediately in three planes using confocal microscopy (Nikon Eclipse TE2000-E, Nikon Corporation, Tokyo, Japan). The images were later analysed for positive staining in all cell nuclei present. Nuclei were excluded from subsequent slices if already counted on a previous slice. To demonstrate the primary antibodies were specific for each cell type, blastocysts were used as a positive control.

**Table 3.1 Species of origin, isotypes, antigenic specificities, dilutions for use and sources of antibodies used in these studies.**

<b>Antibody</b>	<b>Species (Isotype)</b>	<b>Antigenic specificity</b>	<b>Dilution</b>	<b>Source</b>
Unconjugated antibodies				
C-terminal affinity purified Cdx2	rabbit (IgG)	Polyclonal	1:100	Gift <sup>1</sup>
Oct-3/4 (N-19)	goat (IgG)	Polyclonal	1:150	Santa Cruz Biotechnology <sup>2</sup>
Fluorophore conjugated secondary antibodies				
FITC-conjugated $\alpha$ -rabbit	donkey		1:200	Jackson ImmunoResearch <sup>3</sup>
TRITC-conjugated $\alpha$ -goat	donkey		1:200	Jackson ImmunoResearch <sup>3</sup>

<sup>1</sup> Kind gift from Professor Tom Fleming and Dr Jane Collins, Division of Infection, Inflammation and Repair, The University of Southampton, Southampton, UK.

<sup>2</sup> Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA.

<sup>3</sup> Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA.

### **3.3 Results**

#### ***3.3.1 Maturation status following IVM***

To determine if varying oxygen levels during IVM had any effect on oocyte nuclear maturation, orcein staining that allows visualisation of the nuclear structures was used after 17 hr maturation. A representative image is shown in Figure 3.1. Oocyte nuclear maturation status was assessed across 5 experimental replicates (total number of oocytes per group  $\geq 34$ ). The number of oocytes that reached MII stage of maturation after IVM ranged from 80-97%, but there were no significant differences in maturation rate following IVM under 2, 5, 10 or 20% oxygen (Table 3.2).

#### ***3.3.2 Fertilisation rate following IVM***

To determine the rate of fertilisation following IVM, IVF was performed followed by orcein staining to assist in the visualisation of the male pronucleus. Male pronucleus formation was assessed over a minimum of 6 replicates ( $n \geq 16$ ). The ability of oocytes to be fertilised by fresh sperm was not significantly affected by the oxygen concentration during IVM, as indicated by male pronucleus formation (Table 3.2). All treatment groups showed an average rate of fertilisation between 87.5 to 95.8%.

#### ***3.3.3 In vitro embryo development following IVM and IVF***

To determine the effect of IVM oxygen concentration on rate of embryo development, following IVF, presumptive zygotes were cultured and stage of embryo development assessed. Development to 2-cell stage was determined across 20 replicates ( $n \geq 419$ ) and blastocyst development was examined over 14 replicates ( $n \geq 240$ ). Oxygen concentration during IVM had no effect on the number of oocytes that cleaved to the 2-cell stage (Table 3.2). Across the groups an average of 82-87% cleavage rate was observed. There

was also no significant difference in the number of cleaved embryos that went on to form blastocysts (Table 3.2).

#### **3.3.4 Blastocyst cell number and allocation following IVM, IVF and IVC**

As oxygen concentration during IVM did not alter rate of blastocyst development, blastocysts were differentially stained to identify the two cellular compartments of the blastocyst, the ICM and TE, to determine if cell number and allocation were altered. Blastocyst total cell number decreased as oxygen during IVM increased (Figure 3.2A). Blastocysts derived from the 2% oxygen matured oocytes had more total cells, when compared to blastocysts from oocytes matured at 10 or 20% oxygen. The numbers of TE cells in the blastocysts also decreased as IVM oxygen level increased (Figure 3.2B). Blastocysts from 2% oxygen matured oocytes had increased numbers of TE cells, compared to all other treatment groups. TE cell number was also higher in blastocysts from 5% oxygen matured oocytes, when compared to the 20% group. Oxygen concentration during IVM did not alter the numbers of ICM cells present in blastocysts (Figure 3.2C). ICM-to-total cell ratio (expressed as a percentage) was lower in blastocysts derived from the 2%, compared to 20% oxygen treated oocytes (Figure 3.2D).

#### **3.3.5 Blastocyst total apoptosis**

Differential staining identifies all cells present in the blastocyst. To determine the number of these cells that were non-viable, TUNEL was used to identify the total amount of apoptotic cells present in the blastocyst following IVC. The average percentage of apoptotic cells was increased in blastocysts derived from oocytes matured at all oxygen levels compared to those derived from oocytes matured *in vivo* (Figure 3.3). The blastocysts from the 2% oocytes also had significantly higher levels of apoptosis than blastocysts from oocytes matured at 5% oxygen.



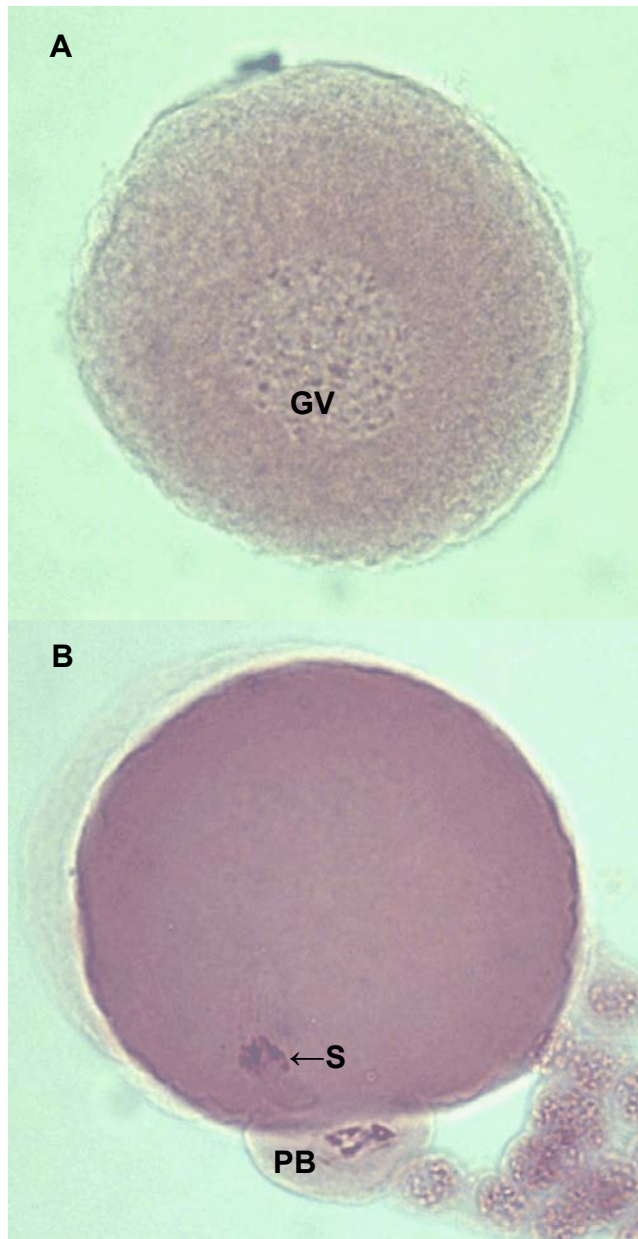
### **3.3.6 Localisation of Cdx2 and Oct4 proteins in morulae**

Cell lineage (ICM or TE) is determined prior to blastocyst formation. Transcription factors Cdx2 and Oct4 were used as markers of TE cell differentiation and ICM cell phenotype, respectively. To determine if more cells were fated to become TE cells in embryos cultured at 2% oxygen, immunohistochemistry was used to localise Cdx2 (Figure 3.6A) or Oct4 (Figure 3.6B) proteins in morulae derived from IVM oocytes (2, 5, 20% oxygen) or *in vivo* matured oocytes. Localisation of Cdx2 was examined over 3 experimental replicates (total number of morulae examined per group  $\geq 7$ ). Cdx2 was located in a significantly higher number of cells in morulae derived from oocytes matured at 2% oxygen compared to those from 5% oxygen IVM and *in vivo* matured oocytes, supporting the differential cell data in 3.3.4. Localisation of Oct4 was analysed across 4 experimental replicates (total number of morulae examined per group  $\geq 20$ ). An increase in the number of cells staining positive for Oct4 was observed in the 2% IVM derived morulae compared to those from 5% oxygen IVM and *in vivo* matured oocytes.

**Table 3.2 The effect of IVM oxygen concentration on maturation, fertilisation, cleavage and blastocyst development rates.**

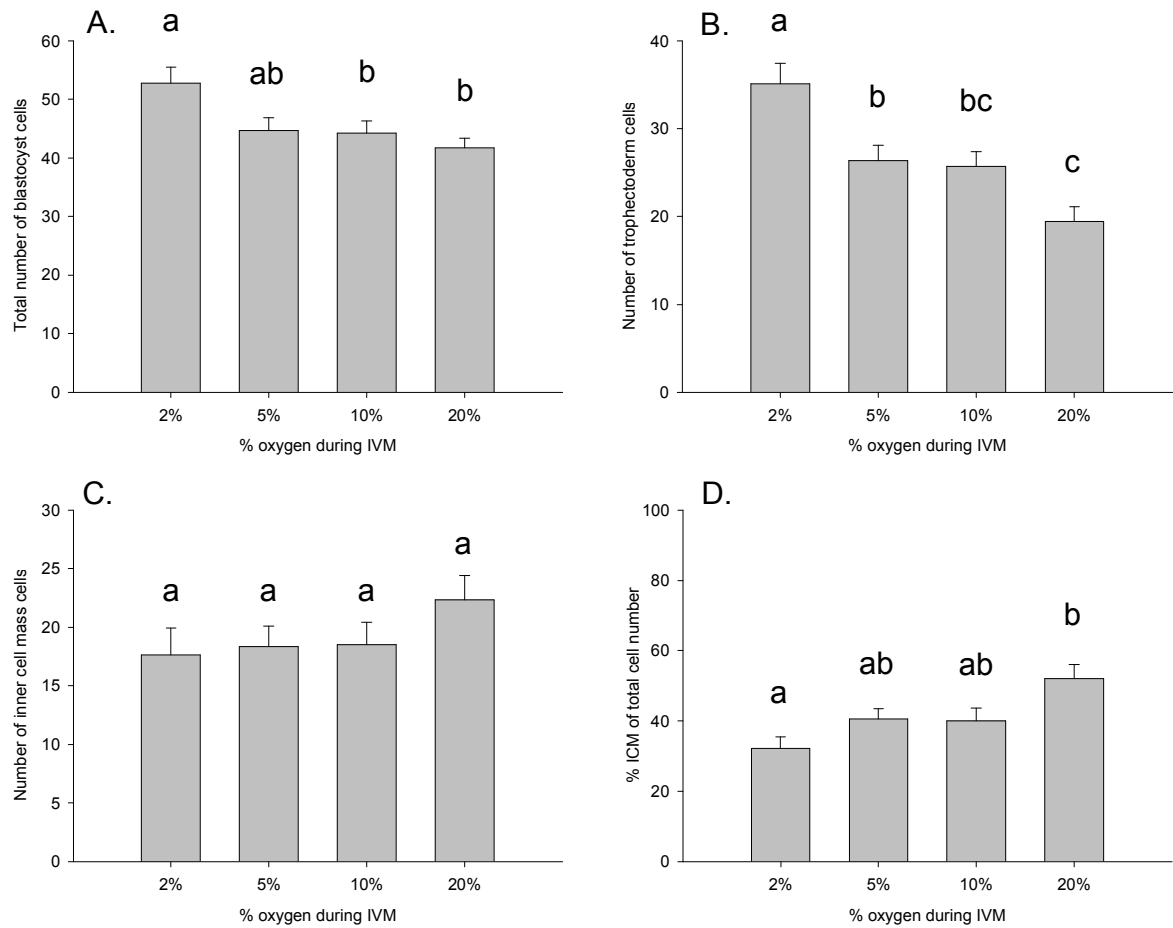
	<b>2% O<sub>2</sub></b>	<b>5% O<sub>2</sub></b>	<b>10% O<sub>2</sub></b>	<b>20% O<sub>2</sub></b>
Oocytes reaching MII (%)	89.0	80.2	97.3	97.4
Male pronucleus formation (%)	88.9	92.3	87.5	95.8
Cleavage to 2-cell (%)	85.2 ± 0.7	86.7 ± 0.6	82.2 ± 0.9	86.5 ± 0.5
Blastocyst development from 2-cell (%)	67.7 ± 4.1	61.9 ± 3.8	64.6 ± 4.2	55.2 ± 3.3

Data are represented as mean (oocytes reaching MII and male pronucleus formation) or mean ± SEM (cleavage to 2-cell and blastocyst development from 2-cell). Oocyte nuclear maturation status was assessed across 5 experimental replicates (total number of oocytes per group ≥34). Male pronucleus formation was assessed over a minimum of 6 replicates (n≥16). Development to 2-cell stage was determined across 20 replicates (n≥419). Blastocyst development was examined over 14 replicates (n≥240).



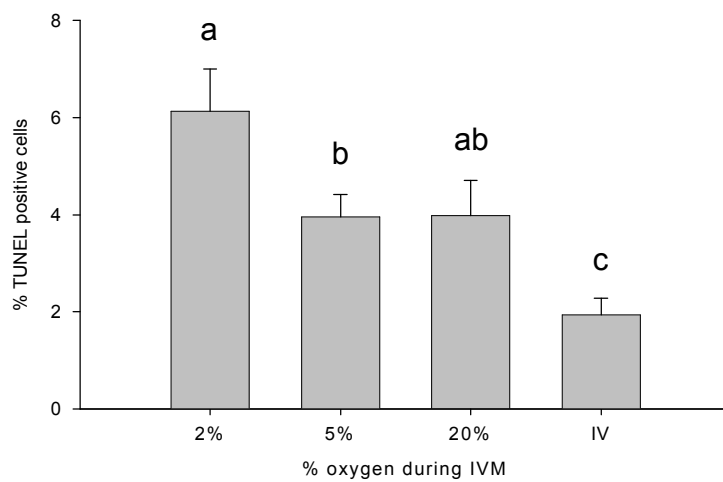
**Figure 3.1 Representative orcein stained oocytes following IVM showing (A) germinal vesicle breakdown and (B) MII spindle plate and first polar body.**

Mouse oocytes were stained with 1% orcein solution to allow visualisation of the nuclear structures. GV, Germinal vesicle; S, MII spindle; PB, polar body.



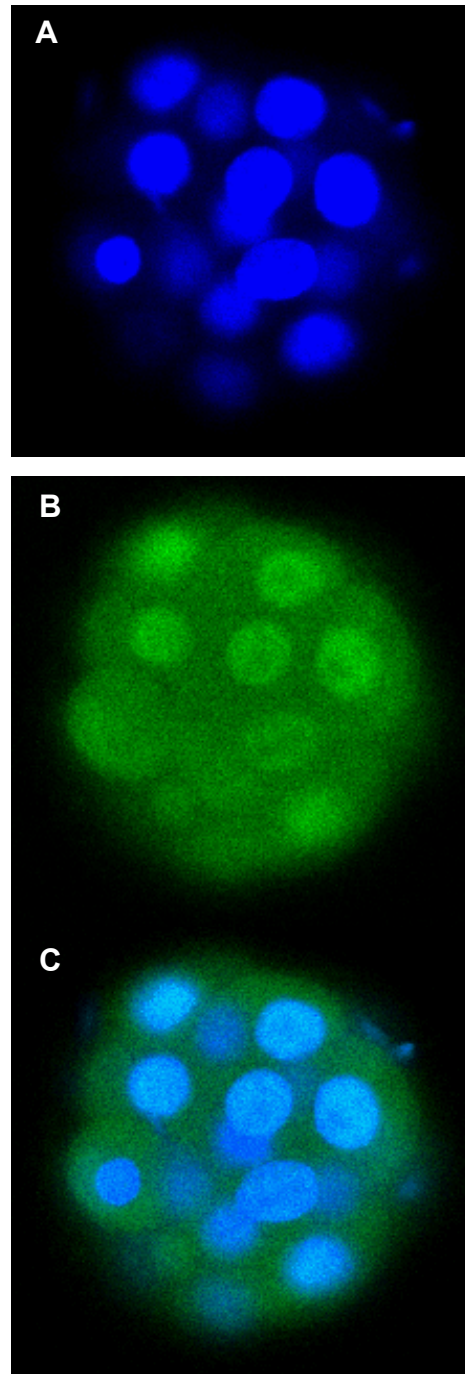
**Figure 3.2 The effects of oxygen treatment during IVM on (A) total number of blastocyst cells, (B) number of trophectoderm cells, (C) number of inner cell mass cells and (D) % inner cell mass cells of total cell number.**

Data are represented as mean  $\pm$  SEM. A minimum of 24 blastocysts were stained for each treatment group across 10 experiments. Different superscripts denote a significant difference between treatment groups ( $P < 0.05$ ).



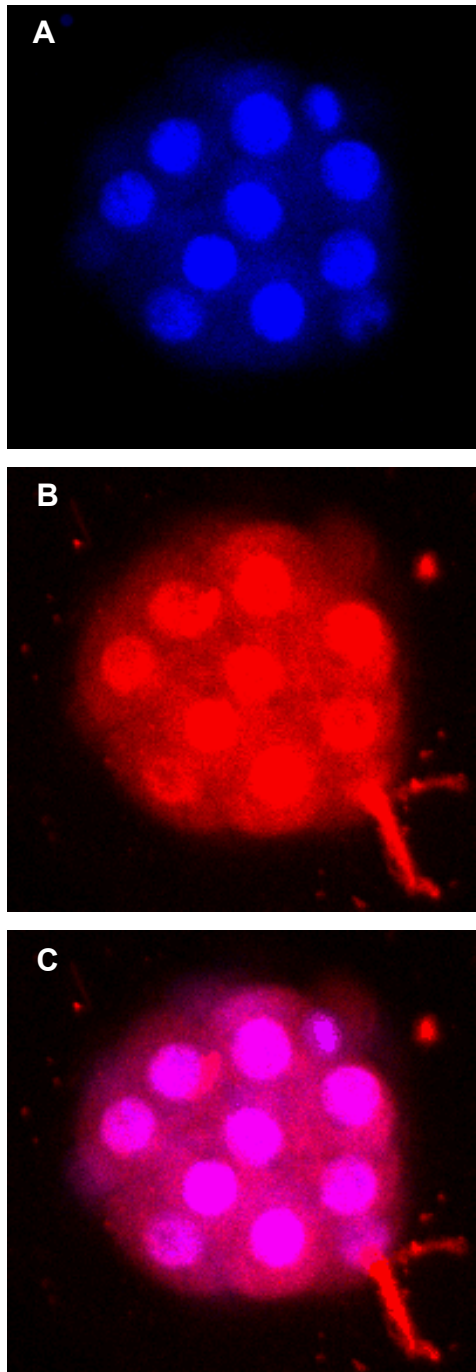
**Figure 3.3 The effect of IVM oxygen conditions in maturation *in vivo* (IV) on the percentage of TUNEL positive cells in the resulting blastocyst.**

Data are represented as mean  $\pm$  SEM. A minimum of 30 blastocysts were assessed for each treatment group across 4 experimental replicates. Different superscripts denote a significant difference between treatment groups ( $P < 0.05$ ).



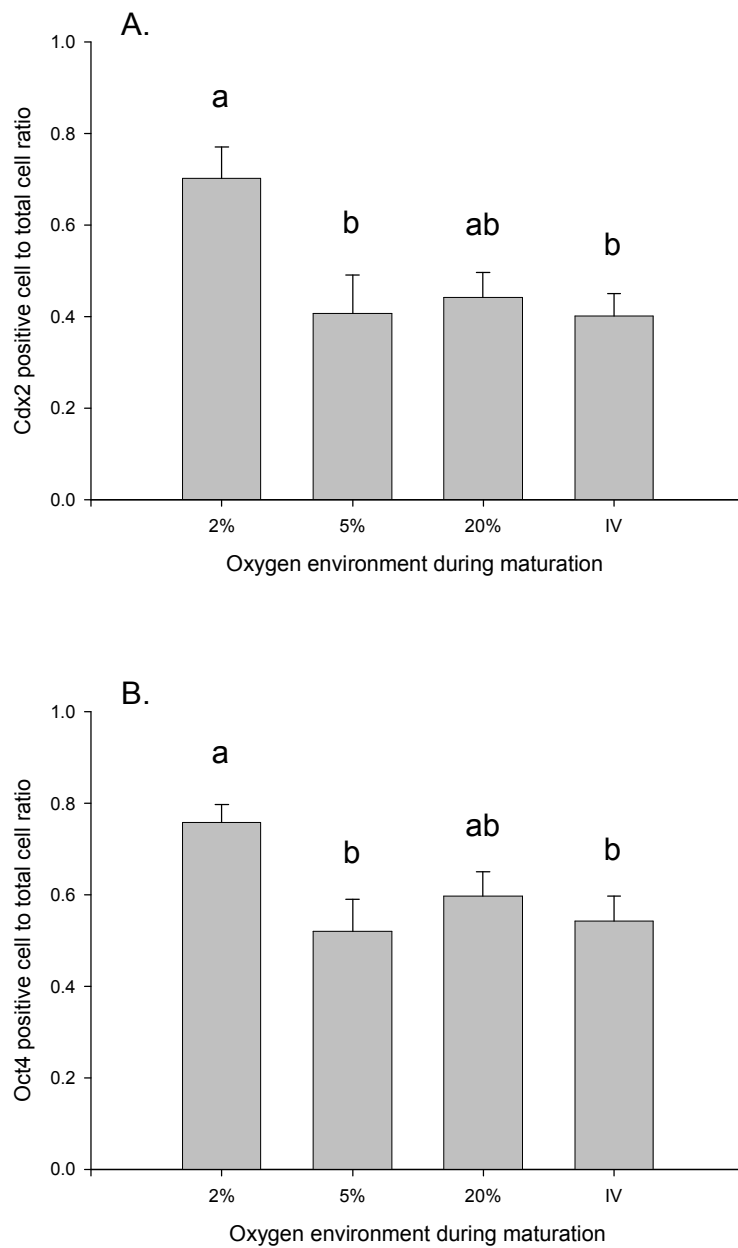
**Figure 3.4 Confocal images illustrating nuclear Cdx2 protein localisation within a murine morulae following IVM under 5% oxygen atmosphere followed by IVF and IVC under 5% oxygen.**

Murine morulae were incubated with primary antibody directed against Cdx2, followed by a FITC-labelled secondary antibody (B: green fluorescence) and DAPI to label nuclei (A: blue fluorescence). Blue and green images are also shown overlaid (C).



**Figure 3.5 Confocal images illustrating nuclear Oct4 protein localisation within a murine morulae following IVM under 2% oxygen atmosphere followed by IVF and IVC under 5% oxygen.**

Murine morulae were incubated with primary antibody directed against Oct4, followed by a TRITC-labelled secondary antibody (B: red fluorescence) and DAPI to label nuclei (A: blue fluorescence). Blue and red images are also shown overlaid (C).



**Figure 3.6 The effect of maturation oxygen environment on the ratio of (A) Cdx2 positive and (B) Oct4 positive cells in murine morulae (in ratio to total cell number) following IVF and IVC.**

Data is expressed as mean  $\pm$  SEM. A minimum of 7 morulae were examined over 3 experimental replicates for Cdx2 localisation. A minimum of 20 morulae were examined over 4 replicates for Oct4 localisation. Different superscripts denote a significant difference between treatment groups ( $P < 0.05$ ).



### 3.4 Discussion

Since the 1970s, the study of oxygen concentration during mouse oocyte maturation has almost exclusively involved the use of 5 or 20% oxygen atmospheres (Eppig and Wigglesworth 1995; Hu *et al.* 2001; Adam *et al.* 2004). This is largely due to previous studies showing that a 5% oxygen concentration is optimal for oocyte nuclear maturation (as measured by extrusion of a polar body) in both mouse and hamster (Haidri *et al.* 1971; Gwatkin and Haidri 1974) and that mouse embryo culture is widely performed under 5% oxygen (Harlow and Quinn 1979; Thompson *et al.* 1990). In the current study I have demonstrated that the oxygen concentration under the conditions used during mouse IVM of COCs, including below 5% oxygen, causes no measurable differences in the ability of those oocytes to reach the MII stage of nuclear maturation, fertilise, cleave or reach the blastocyst stage of embryo development. However, the oxygen environment during IVM does affect the cellular development of resulting blastocysts, revealing for the first time that oxygen concentration during IVM affects subsequent blastocyst cell lineage in the mouse.

Total blastocyst cell number decreased as oxygen concentration during oocyte maturation increased. This change in blastocyst total cell number can be attributed to a reduction in the number of TE cells with increasing oxygen levels. Although apoptosis rates were also higher in blastocysts derived from oocytes matured at 2% oxygen, this increase in total cell number was not fully accounted for by the presence of apoptotic cells, as TUNEL only detected a small number of such cells. Higher total cell number was also observed in porcine blastocysts following IVM of oocytes under 5% oxygen and electrical pulse activation or fertilisation with frozen sperm (Kikuchi *et al.* 2002; Iwamoto *et al.* 2005). However, an increased activation rate in the 5% oxygen matured oocytes was also observed (Iwamoto *et al.* 2005). This is in contrast to our study, which found no significant effect of oxygen environment during oocyte maturation on fertilisation rates with fresh sperm. Significantly, work by Preis, Seidel and Gardner (2007) also found similar rates of blastocyst formation regardless of IVM oxygen level, with increased cell number following IVM under 5%

oxygen compared to 20%. However, the cell type responsible for this increase was not determined in this study, nor was an oxygen level below 5% examined for comparison. This highlights how the conclusions drawn from a study can be influenced by comparison of a limited number of treatments and/or the breadth of outcome measures.

The expression of *Cdx2*, a caudal-type homeodomain transcription factor, is required for trophoctoderm development in mice and is used as a trophoctoderm cell marker (Strumpf *et al.* 2005; Tolkunova *et al.* 2006). There is some debate as to the exact point in development *Cdx2* becomes expressed exclusively by cells destined to become trophoctoderm, however, it occurs prior to blastocyst formation at a time when there is no obvious marker of cell type (Ralston and Rossant 2007). *Oct4*, a common marker of pluripotency, becomes restricted to cells of the developing embryo destined to become inner cell mass cells while *Cdx2* becomes increasingly restricted to the trophoctoderm (Strumpf *et al.* 2005). The localisation of *Cdx2* and *Oct4* in morulae derived from oocytes matured under different oxygen concentrations was examined to determine if more of the cells under low oxygen were destined to become trophoctoderm prior to blastocyst development. The increased ratio of cells positive for *Cdx2* in morulae derived from IVM at 2% oxygen seemed to suggest this was the case. However, morulae derived from IVM at 2% oxygen also had an increased ratio of cells positive for *Oct4*.

As *Oct4* is a common marker of pluripotency it should only be expressed in cells that are not yet terminally differentiated. More cells in morulae derived from low oxygen matured oocytes had yet to switch off *Oct4* and this suggests (in a mouse model) a retardation of terminal differentiation. Aberrant *Oct4* expression has been documented in trophoblast stem (TS) cells when methylation is inhibited (Okazaki and Maltepe 2006). When this co-localisation of cell markers in the low oxygen morulae is taken together with cell allocation data at blastocyst stage, it is clear that these undecided cells would most likely go on to form TE cells. However, the mechanism by which the increase in trophoctoderm cell number occurs following low oxygen

oocyte maturation was not clearly elucidated within this study. Furthermore, we have not characterised trophectoderm cell numbers following *in vivo* oocyte maturation followed by IVF and so cannot determine which cellular pattern is more like that found *in vivo*.

Trophectoderm cells form the placental interface and are therefore involved in oxygen and nutrient transport to the fetus. Oxygen concentration is a significant regulator of trophectoderm cell differentiation and proliferation (Caniggia and Winter 2002). Nevertheless, blastocyst cell numbers are lower when mouse embryos are cultured post-compaction in 2% oxygen compared to 7% oxygen, which is also reflected in the differential expression of oxygen-sensitive genes in resulting blastocysts (Kind *et al.* 2005). However, whether 2% oxygen represents a hypoxic stimulus for the mouse oocyte is uncertain, as no studies have investigated the *in vivo* follicular oxygen concentration in the mouse. Data from human, bovine and porcine follicles, suggests 2% oxygen would be considered in the low range (Knudsen *et al.* 1978; Van Blerkom *et al.* 1997; Huey *et al.* 1999; Haddad 2002; Berg *et al.* 2003). Conversely, 20% oxygen represents a non-physiological oxygen condition, and it is also possible that an increase in oxidative stress during IVM in the 20% group may contribute to the alteration in cell fate programming.

ICM number was not significantly altered following IVM at different oxygen concentrations; however the decrease in total cell number resulted in an alteration of the ICM-to-total cell ratio. The total blastocyst cell number and the number of ICM cells have been reported as positively correlated with fetal development after embryo transfer (Lane and Gardner 1997). The cell number results in the current study therefore suggested that embryos derived from oocytes matured at different oxygen levels may have differential developmental capacity once transferred, perhaps decreasing with increased oxygen levels.

*In vivo*, the oocyte is buffered from the external environment by the FF and other cellular follicular components. During IVM, the surrounding cumulus cells also buffer the oocyte from the external environment. Using mathematical modelling and assumptions based on *in vitro*-derived data, it has been

calculated that little oxygen is lost through diffusion across the cumulus cell layer that surrounds the oocyte, with the mouse cumulus mass consuming approximately 0.5% oxygen when the FF oxygen concentration is in the range of 3 to 20% (Clark *et al.* 2006; Redding *et al.* 2007). Therefore it is unlikely that an anoxic state was observed at the surface of the oocyte under the conditions utilized here. However, I have yet to determine, under these incubation conditions, if oxygen concentration is rate-limiting for oxidative phosphorylation, especially at the lower concentrations. Further studies will be aimed at investigating ATP production and mitochondrial function and location within oocytes matured under differing oxygen concentrations.

Overall my results demonstrate that in the system used here, oxygen concentration during IVM had no effect on maturation or fertilisation rate, or subsequent cleavage and development to blastocyst. However, as oxygen level during maturation decreased, blastocyst trophoctoderm cell number increased, and while the amount of apoptosis also increased, this was not of significant magnitude to counteract this change in cell number. Furthermore, morulae derived from oocytes matured at 2% oxygen exhibited a retardation of terminal differentiation as evidenced by an increased number of cells expressing Oct4. These results were obtained using one particular mouse model. Unfortunately, this is a limitation as we have not investigated species differences or effect of alternative culture system. Nonetheless, increased understanding of how the further development of the oocyte is altered across a broad range of oxygen environments during this critical period of oocyte maturation adds to the scientific knowledge in this area. As a whole, this data suggests the exclusion of low oxygen concentrations (< 5%) in studies using current IVM systems is unfounded and further work in this area should include a broader range of oxygen treatments to provide a more complete picture.

## **4 Oxygen during in vitro maturation; effects on fetal and placental development**

## 4.1 Introduction

IVM has been proposed as a possible adjunct therapy to traditional IVF for infertile couples, especially for women who respond inappropriately to gonadotrophin stimulation or as an alternative to stimulation for all patients. However, few pregnancies resulting from human oocyte IVM have been recorded and the process requires further improvement and assessment before becoming routine clinical practice (Trounson *et al.* 1994; Barnes *et al.* 1996; Cha and Chian 1998; Cha *et al.* 2000; Kim *et al.* 2000; Smith *et al.* 2000). This is mirrored by results in most other mammalian species, where it is widely accepted that oocytes matured *in vitro* have a reduced developmental capacity when compared to *in vivo* matured oocytes (Eppig and Schroeder 1989; Combelles *et al.* 2002).

It has been shown that a high grade of follicular vascularity (as assessed by Doppler ultrasonography) correlates with a higher rate of pregnancy and live births following embryo transfer in women (Chui *et al.* 1997), suggesting that this is important for successful development. The lack of vasculature inside the ovarian follicle means oxygen must diffuse across the follicle wall and fluid filled antrum to be available for use by the oocyte and the surrounding cumulus cells.

It was found in Chapter 3 that oxygen concentration (either 2, 5, 10 or 20% O<sub>2</sub>, 6% CO<sub>2</sub> and balance of N<sub>2</sub>) during IVM of murine COCs had no effect on maturation rate or subsequent fertilisation of oocytes, cleavage and blastocyst development rates. However, 2% oxygen during IVM results in blastocysts with a higher ( $P < 0.05$ ) trophectoderm cell number (mean  $\pm$  SEM,  $35.1 \pm 2.3$ ) when compared to 20% ( $19.4 \pm 1.7$ ), with 5 and 10% oxygen yielding intermediate cell numbers. An increase in cell apoptosis levels under low oxygen (2%) was not sufficient to counteract this increase in cell number. Changes in cell number have been correlated with fetal development following transfer (Lane and Gardner 1997). The presence of ammonium in mouse embryo culture medium (during precompaction stages and all the way through culture) resulted in blastocysts with a decreased ICM cell number and a reduced total cell number (Zander *et al.*

2006). Once transferred, these blastocysts exhibited a decreased percentage of viable fetuses per embryo transferred and per implantation as well as a reduction in fetal maturity (Zander *et al.* 2006). Likewise, some stimulation protocols shown to reduce blastocyst cell number (Edwards *et al.* 2005) also cause perturbations in pregnancy outcomes (Ertzeid and Storeng 2001). In cattle where culture perturbations (such as the presence of serum) result in heavier offspring (LOS), this is also associated with increased blastocyst cell number prior to transfer (Lazzari *et al.* 2002). This evidence suggests blastocyst cell number and fetal outcomes are linked. Our data therefore suggests that IVM oxygen concentration, with a demonstrated effect on blastocyst cell number and allocation, may also have implications for pregnancy outcomes following embryo transfer. This has not been previously studied.

Accordingly, we investigated the impact of oxygen concentration during mouse IVM on subsequent pre-implantation embryo development and post-transfer outcomes. The range of oxygen concentrations studied included 2, 5 and 20% oxygen mixtures. The 10% IVM oxygen group was excluded due to the difficulty in achieving an adequate number of pregnancies and pups from IVM embryos following transfer overall and because of the similarity of the previous results to the 5% oxygen treatment. Post-transfer measures included implantation and development, fetal crown-rump length and fetal and placental weights.

## **4.2 Materials and methods specific to this chapter**

Mouse ovarian stimulation, oocyte collection, IVM, IVF and IVC were performed as outlined in Chapter 2.

### **4.2.1 Embryo transfer**

To determine if IVM oxygen concentration had any effect on the ability of the subsequent embryo to implant and form a viable pregnancy, blastocysts were transferred into the uterus of recipient mice and post-mortem examination of the uterus was performed on day 18 of pregnancy (term = day 19-20). Naturally ovulating female Swiss mice aged between 8 and 12 weeks were mated with vasectomized CBAB6F1 males to

induce pseudopregnancy. On day 4 of pseudopregnancy the female recipients were anaesthetised with 2% Avertin (2,2,2-tribromoethanol in 2-methyl-2-butanol, diluted to 2% in sterile saline; 0.015 ml/g body weight) and 6 embryos transferred to each uterine horn (approximately 90 h post-fertilisation). Treatment groups were randomly allocated to mouse and horn by random number selection. The absence of transuterine migration following embryo transfer in mice has been verified in recent studies (Rulicke *et al.* 2006).

#### **4.2.2 Fetal and placental analysis at day 18 of pregnancy**

To determine the effects of IVM oxygen concentration on subsequent fetal and placental parameters following transfer, post-mortem examination of the uterus was performed on day 18 of pregnancy. Embryo transfer recipients were sacrificed on day 18 of pregnancy by cervical dislocation. A post-mortem examination was carried out to determine the number of implantations and fetuses, as well as fetal and placental weight and fetal crown-rump length. Briefly, the uterus was removed from the recipient with care to keep the horns in the correct orientation and placed on a tray in the freezer for 5 minutes. Each horn of the uterus was dissected in turn and examined to identify the number of both viable and non-viable conceptuses present. Each fetal unit was excised from the uterus, debris including the umbilicus was removed, and the fetus weighed. The placenta was then removed from the membrane and weighed. Each fetus was measured from crown to rump with a set of callipers.

### **4.3 Results**

#### **4.3.1 Implantation outcomes following embryo transfer**

To determine the effect of oxygen during maturation on post-implantation outcomes, embryos were transferred to a total of 33 recipients with 6 embryos transferred per horn. Twenty-two of the recipients were pregnant in at least one horn at day 18 of pregnancy. Non-pregnant animals contained embryos from all treatment groups (2%: n = 7 horns; 5%: n = 6 horns; 20%: n = 5 horns; *in vivo*: n = 2 horns). These animals



were not included in further analysis. A total of 43 uterine horns were studied (2%: n = 11; 5%: n = 12; 20%: n = 10; *in vivo*: n = 10; one horn in one animal did not receive transfer due to uterine horn malformation). Blastocysts were generated from at least 5 experiments. No implantation sites were detected in 3 of the horns studied (pregnant animals) (2%: n = 1; 5%: n = 2). Implantation rate and development of a viable fetus following implantation (viable fetuses/blastocyst implanted) was not different between oocyte maturation groups or when compared to blastocysts derived from *in vivo* matured oocytes (Table 4.1).

#### **4.3.2 Fetal outcomes following embryo transfer**

To determine if IVM oxygen concentration altered fetal parameters, conceptuses present in the uterus on day 18 of pregnancy were assessed. Viable conceptuses present within a uterine horn at day 18 of pregnancy ranged from 1 to 5 (mean,  $1.7 \pm 0.2$ ). Total number of fetuses carried by the recipient mothers ranged from 1 to 6 (mean,  $3.3 \pm 0.3$ ) and the total number of implantations per mother ranged from 2 to 12 (mean,  $7.6 \pm 0.7$ ). The number of viable fetuses at day 18 of pregnancy within a horn did not vary with treatment (2%,  $1.7 \pm 0.5$  (n = 11 horns); 5%,  $1.8 \pm 0.4$  (n = 12 horns); 20%,  $1.9 \pm 0.4$  (n = 10 horns); *in vivo*,  $1.6 \pm 0.5$  (n = 10 horns)). Total number of fetuses carried by the mother was also calculated to provide an indication of total maternal load (mothers carrying a horn containing fetuses from 2% matured oocytes: fetal load,  $3.4 \pm 0.4$ , 5%:  $3.4 \pm 0.4$ , 20%:  $3.4 \pm 0.3$ , *in vivo*:  $3.6 \pm 0.4$ ).

IVM at 2, 5 or 20% oxygen did not alter fetal weight, compared to those oocytes matured *in vivo* (Table 4.1). Nevertheless, fetal weight was reduced following IVM at 5% oxygen, compared to the 20% oxygen group (Table 4.1). This difference in fetal weight was independent of the number of fetuses or implantations within the treatment horn, or the total number of fetuses or implantations within the mother. Fetal crown-rump length was not altered following maturation at any oxygen concentration, when compared across treatment groups, or to those matured *in vivo*.

### **4.3.3 Placental outcomes following embryo transfer**

To determine if IVM oxygen concentration altered placental parameters, conceptuses present in the uterus on day 18 of pregnancy were assessed. Placental weight was reduced following IVM at 5% oxygen, compared to placentae derived from *in vivo* matured oocytes (Table 4.1). This difference in placental weight was independent of the number of implantations within the treatment horn, or total litter size, but was not significant when corrected for the number of fetuses within the treatment horn. The fetal: placental weight ratio was not altered by maturation oxygen environment.

**Table 4.1 The effect of IVM oxygen concentration on implantation rates and subsequent fetal and placental development.**

	<b>2% O<sub>2</sub></b>	<b>5% O<sub>2</sub></b>	<b>20% O<sub>2</sub></b>	<b><i>In vivo</i></b>
Implantation rate <sup>A</sup> (%)	69.7 ± 10.5	56.9 ± 9.0	80.0 ± 5.4	65.0 ± 10.1
Viable fetuses/blastocyst implanted <sup>B</sup> (%)	45.0 ± 10.3	49.3 ± 8.8	42.0 ± 9.0	39.7 ± 10.4
Fetal weight (mg)	870.2 ± 26.7 <sup>ab</sup>	823.3 ± 28.1 <sup>a</sup>	928.5 ± 26.1 <sup>b</sup>	879.3 ± 32.3 <sup>ab</sup>
Fetal crown-rump length (mm)	18.4 ± 1.0	19.9 ± 0.3	20.1 ± 0.3	19.3 ± 0.4
Placental weight (mg)	98.7 ± 5.5 <sup>ab</sup>	87.4 ± 4.0 <sup>a</sup>	100.1 ± 5.5 <sup>ab</sup>	104.5 ± 5.4 <sup>b</sup>
Fetal : placental weight ratio	9.1 ± 0.4	9.8 ± 0.5	9.6 ± 0.5	8.8 ± 0.5

Number of blastocysts transferred, 2% n=60; 5% n=72; 20% n=60; *in vivo* n=60. <sup>A</sup>Implantation rate: number of fetal/placental units or resorptions present as a percentage of the number of embryos transferred. <sup>B</sup>Viable fetuses/blastocyst implanted: percentage of implanted embryos that developed into a viable fetus (2%, n=19; 5%, n=21; 20%, n=19; *in vivo*, n=16). Different superscripts within a row represent statistically significant differences, P < 0.05.

## 4.4 Discussion

In Chapter 3 it was shown that ICM number was not significantly altered following IVM at different oxygen concentrations; however the decrease in total cell number with increasing oxygen levels resulted in an alteration of the ICM-to-total cell ratio. In many systems, change in blastocyst cell number has been linked with differences in fetal outcomes following transfers (Lane and Gardner 1997; Ertzeid and Storeng 2001; Lazzari *et al.* 2002; Zander *et al.* 2006). My results therefore suggested that embryos derived from oocytes matured at different oxygen concentrations may have differing potential following transfer; specifically, that embryos from 2% oxygen matured oocytes may have a reduced developmental capacity. However, regardless of the oxygen environment during maturation, the subsequent blastocysts were able to implant and develop at the same rate as those derived from oocytes matured *in vivo*. The number of total cells present in the blastocyst at the time of transfer did not, in this case, correlate with implantation success.

Maturation under 5% oxygen gave rise to fetuses that were significantly lighter than those derived from 20% oxygen matured oocytes. This suggests a more adverse outcome following IVM under 5% oxygen in terms of fetal weight, however, these weights were not different from those resulting from fetuses derived from *in vivo* oocyte maturation. The placentae from 5% oxygen matured oocytes also weighed less and in this instance they were significantly different compared to *in vivo* controls. Although the structure and function of the placentae has not been investigated here, there was no alteration in the fetal-to-placental weight ratio, suggesting that placental function may not have been altered.

Across all our comparisons to the *in vivo* matured oocyte and subsequent embryo and conceptus development, we could find no evidence that any of our treatments resulted in poorer outcomes when compared to oocytes matured *in vivo*. Contrary to the current dogma, we found little evidence that a low level of oxygen during the final stages of oocyte maturation has any sustained detrimental effect on outcomes for the conceptus. Nevertheless, although 2% oxygen is considered a moderate hypoxic state,

lower oxygen concentrations may indeed induce more detrimental short- and long-term outcomes. Longer term post-natal outcomes following IVM are not addressed by this study, and have been little studied by others. The longer term outcome for offspring following IVM should be investigated as it is possible the short term differences seen in this study will translate to effects in adulthood as is the case with other reproductive technologies (Thompson *et al.* 2007).

Low oxygen availability in the follicle has been shown to correlate with increased oocyte chromosomal abnormalities and decreased pregnancy rate (Chui *et al.* 1997; Van Blerkom *et al.* 1997). The exposure of an oocyte to a low oxygen environment during follicular growth is likely to be longer than the relatively short exposure during IVM (17 h). Therefore, it remains to be determined if detrimental effects on long-term outcomes may be more dramatic from a chronic exposure to low oxygen. Another consideration that Hashimoto *et al.* (2000a) revealed is that cattle oocyte developmental potential when matured under different oxygen concentrations depended on the level of glucose availability. We cannot rule out that our results may have differed if an alternative IVM system had been utilised.

Finally, all of the oocytes used in this study were *in vitro* fertilised and the resulting zygotes cultured. It is known that the embryo culture environment can affect the subsequent long-term outcomes (Fleming *et al.* 2004; Thompson *et al.* 2007). In this study, relatively small differences were observed between embryos derived from *in vivo* matured oocytes and those from IVM. Therefore it is plausible that developmental potential of blastocyst-stage embryos is primarily determined by the culture environment during embryo development in comparison with conditions used during IVM. This could be examined if we had compared the survival amongst *in vivo* derived blastocysts with the treatments outlined here.

For IVM to become routine clinical practice as an addition to human infertility treatment, such as IVF, the effects of this additional manipulation must be fully understood and the environment optimised. Indeed, even when used in an experimental setting, it is important to comprehend the changes induced in outcomes

by environmental factors. The results of my studies demonstrate that the oxygen environment under which the oocyte is matured can alter cell number in resulting blastocysts, which appear to not correlate well with the subtle changes in subsequent fetal and placental parameters. This suggests that changes to the embryo development induced by IVM oxygen concentration are not necessarily reflected in altered fetal outcomes following transfer. If this study had followed the usual paradigm of simply comparing 5% vs 20% (or air) oxygen atmospheres during IVM, very different conclusions would have been reached. The data obtained under this circumstance would have led to conclusions that differences in cell allocation in blastocysts correlated to differences in fetal and placental weight, suggesting in general that low oxygen (i.e. 5%) during IVM are not suitable for subsequent development. This study further illustrates the value of an expanded comparison when determining the effect of environmental parameters on developmental outcomes.

## **5 Oxygen during in vitro maturation; effects on the COC**

## 5.1 Introduction

In previous chapters, it was proposed that the oxygen concentration during IVM would influence the future developmental competence of the oocyte. In Chapter 3, the ways in which oxygen influenced the early development of the embryo were examined. Although cellular alterations were found in blastocysts derived from different oxygen treatments during IVM, this was not associated with pregnancy outcome following transfer (Chapter 4). However, IVM at 5% oxygen did result in a lighter conceptus, suggesting that oxygen concentration was indeed able to influence fetal and placental parameters, specifically when used at this concentration, although these effects appear unrelated to the cellular alterations in the blastocyst.

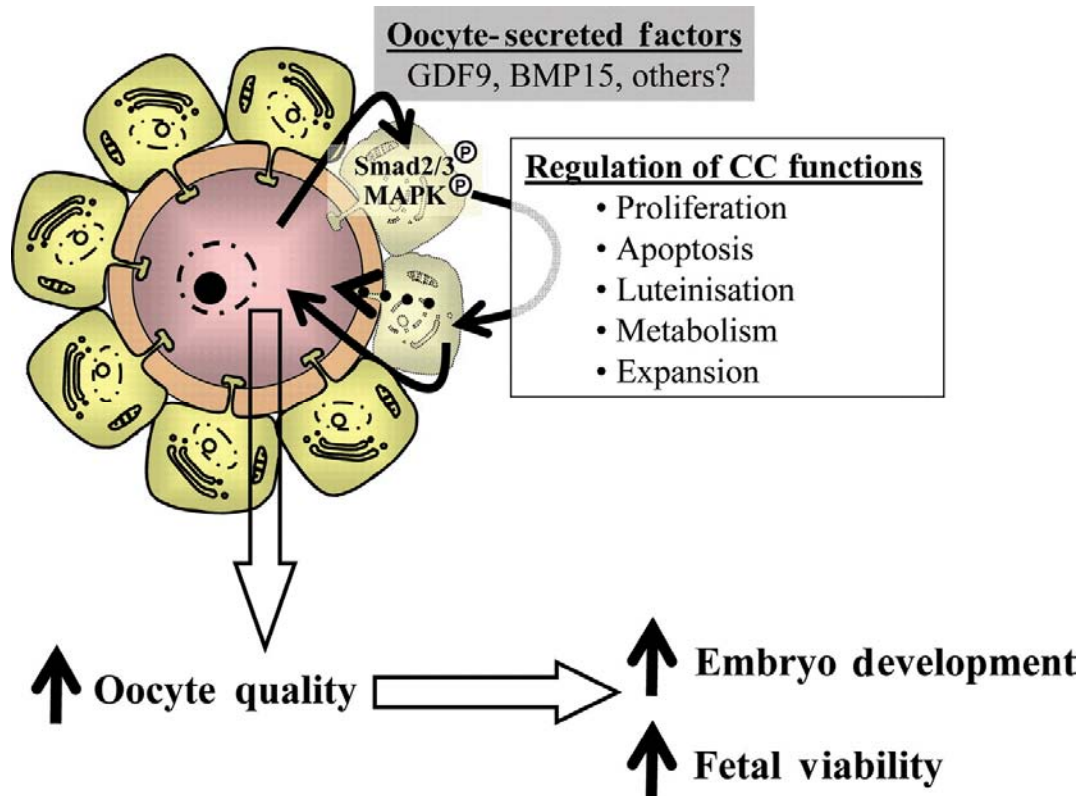
As mentioned previously, the mitochondria are the organelles in the oocyte responsible for oxidative phosphorylation, a process that provides the oocyte energy and is reliant on oxygen availability. Mitochondrial parameters in early development have been studied in many species [mouse; (Bavister and Squirrell 2000; Van Blerkom *et al.* 2002), human; (Wilding *et al.* 2001; Van Blerkom *et al.* 2002), hamster; (Barnett *et al.* 1996) and bovine; (Stojkovic *et al.* 2001)]. Most agree that the distribution and activity of the mitochondria in oocytes is heterogeneous (Stojkovic *et al.* 2001; Wilding *et al.* 2001; Van Blerkom *et al.* 2002). Van Blerkom and colleagues (2002; 2003) identified a patterning of highly active mitochondria in the outer or pericortical region in mouse oocytes. They have speculated that this pattern of increased activity close to the plasma membrane could be caused by the increased oxygen availability at the cell surface (Van Blerkom *et al.* 2002). It has also been speculated that this patterning of highly active mitochondria in the subplasmalemmal cytoplasm has a developmentally relevant purpose; perhaps fulfilling the energy requirements of activities taking place in the cytoplasm (Barnett *et al.* 1996; Van Blerkom *et al.* 2003; Van Blerkom 2004).

As respiration involves outward proton pumping across the mitochondrial membrane causing a proton gradient, the inner mitochondrial membrane potential ( $\Delta\psi_m$ ) is a measure of mitochondrial activity with



differences in  $\Delta\psi_m$  related to levels of respiration (Van Blerkom 2004). The most widely used way to measure mitochondrial activity via membrane potential is by using the compound 5,5',6,6'-tetrachloro-1,1,3,3'-tetraethylbenzimidazolycarbocyanine iodide (JC-1) which exists in a green fluorescence emitting monomer at low potential ( $\leq -100\text{mV}$ ) and forms an aggregate at high potential ( $\geq -140\text{ mV}$ ) with an emission profile in the red spectrum. This molecule has been used to examine the association between mitochondrial activity and developmental competence of the oocyte and early embryo. Changes in the spatial distribution and polarity of mitochondria in the oocyte have been associated with decreased developmental competence (Wilding *et al.* 2001; Van Blerkom *et al.* 2002; Jones *et al.* 2004). Although the process of oxidative phosphorylation is an oxygen dependent process occurring in the oocyte mitochondria, the effect of different oxygen concentrations during oocyte maturation on the patterning and activity of oocyte mitochondria has not been investigated.

Another factor worth consideration is that the oocyte matures in the presence of associated cumulus cells. The cumulus cells and the oocyte share a close relationship with contact via the cumulus cell trans-zonal cytoplasmic projections that form gap junctions at the oocyte surface (Albertini *et al.* 2001). It is through these gap junctions that the cumulus cells are able to provide nutrients and factors to the oocyte. In turn, the oocyte secretes OSFs, which can regulate cumulus cell function (reviewed by Gilchrist *et al.* 2008, see also Figure 5.1 ). It is widely accepted that this relationship is beneficial for further development with removal of cumulus cells before maturation resulting in decreased developmental competence (Vanderhyden and Armstrong 1989; Zhang *et al.* 1995).



**Figure 5.1 Model depicting the interaction between the cumulus cells and the oocyte and the potential impact on the subsequent development of that oocyte.**

An oocyte–somatic cell regulatory loop exists, whereby oocytes and cumulus cells regulate each others functions by paracrine and gap-junctional communication. Oocytes secrete soluble growth factors (like GDF9 and BMP15), that lead to the activation of signalling pathways in the cumulus cells. This in turn, regulates cumulus cell gene expression and multiple cumulus cell functions. Likewise, cumulus cells pass growth factors and small metabolites back to the oocyte via these same systems. This communication loop appears to regulate unknown processes in the oocyte that improves its quality, as assessed by improved developmental potential. Figure reproduced with permission [Gilchrist *et al* (2008)].

Due to this close relationship, events altering the health of the cumulus cells are likely to impact the oocyte and vice versa. Cumulus cell function may therefore provide an indication of oocyte health. Cumulus cells are also normally discarded during IVF cycles, representing a useful supply of material for diagnostics. This opportunity has been recognised by some IVF clinics with the gene expression profile of discarded human cumulus cells investigated as a means of predicting oocyte quality. Expression of some genes, such as gremlin (*GREM1*) and pentraxin 3 (*PTX3*), exhibit this association with oocyte development (McKenzie *et al.* 2004; Zhang *et al.* 2005).

Considering the close and dependent relationship between cumulus cells and the oocyte during maturation, it would be remiss of this study not to consider the impact of altering oxygen concentration on cumulus cells. This has not been previously investigated. Microarray technology was therefore used to investigate the effects of the exposure to differing oxygen concentrations on cumulus cell gene expression. As the effects of oxygen concentration on cumulus cells are largely unknown, this approach will provide an inclusive picture of changes in gene expression between samples without the need for targeted primers (as is the case with PCR).

Both oocyte metabolism and the co-dependent relationship within the COC represent possible pathways by which oxygen concentration during oocyte maturation could influence future development. With this in mind, the aim of our next study was to investigate the effects of oxygen concentration during oocyte maturation on both oocyte mitochondrial parameters and on cumulus cell gene expression.

## **5.2 Materials and methods specific to this chapter**

Mouse ovarian stimulation, oocyte collection, IVM, IVF and IVC were performed as outlined in Chapter 2.

### **5.2.1 Oocyte mitochondrial membrane potential ( $\Delta\psi_m$ ); JC-1 staining**

To determine the effect of maturation under different oxygen concentrations on mitochondrial membrane potential (a common measure of mitochondrial activity) of the oocyte, oocytes matured under 2, 5, 10 and 20% oxygen environments were stained with JC-1 (Molecular Probes), a dual emission, mitochondrial specific stain that allows visualisation and quantification of mitochondrial membrane potential. This is possible through the formation of 'J-aggregates' when the compound is taken up by highly polarised mitochondria, which causes a shift in emission wavelength from green to red. The level of fluorescence in these two spectra can be quantified and expressed as a ratio of active to non-active mitochondria.

COCs were treated with hyaluronidase as described in 3.2.1 to remove cumulus cells. Denuded oocytes were washed through fresh HEPES buffered  $\alpha$ MEM and then placed in 1.5 mM JC-1. Oocytes were incubated for 15 mins at 37 C in the dark before being immediately loaded in confocal loading medium and imaged using confocal microscopy.

#### **5.2.1.1 Analysis of oocyte mitochondrial membrane potential**

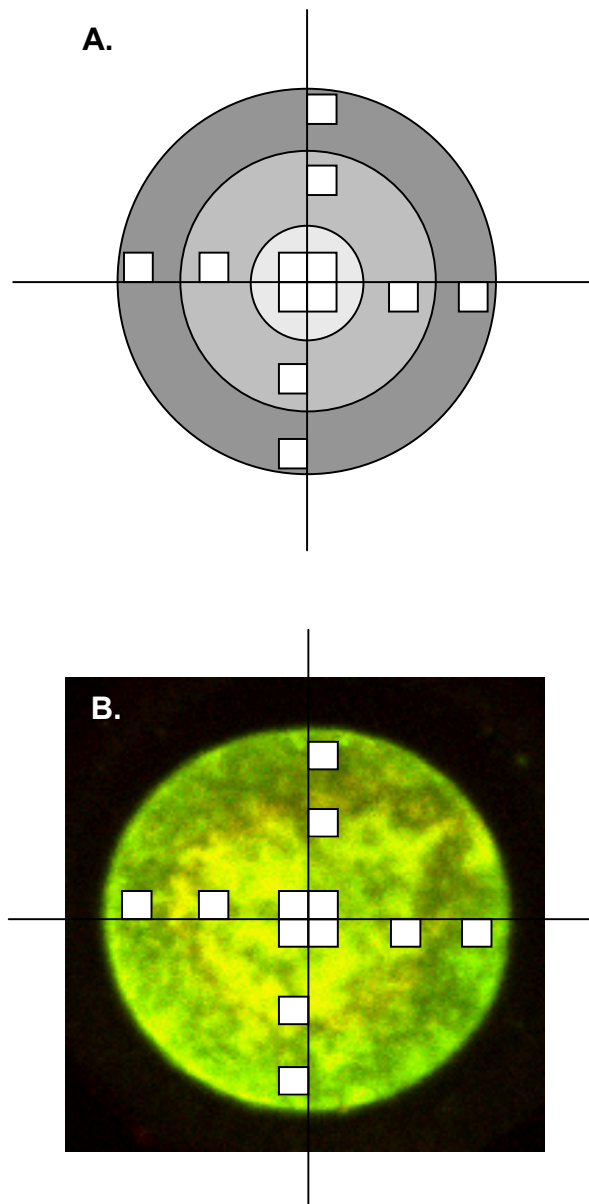
Images were assessed using Adobe Photoshop (version 6.0.1; Adobe Systems Inc., San Jose, USA) to calculate the mean pixel intensity of both the red and green fluorescence in different areas of the oocyte. This was performed by placing an axis over each image, and using the drawing tool to make a box from which a reading of both green and red fluorescence was taken. Readings were taken within each of the three areas (peri-nuclear, intermediate and cortical) along each of the four arms of this axis as shown in Figure 5.2. All readings were taken using square boxes of equal pixel area (111 x 111 pixels) which was kept constant across all oocytes analysed and all images were analysed at the same magnification.

## **5.2.2 Oocyte mitochondrial distribution; MitoTracker Green staining**

To determine if the location of all mitochondria across the ooplasm was affected by maturation in different oxygen environments, oocytes matured under 2, 5 and 20% oxygen and matured *in vivo* were assessed using MitoTracker Green (Molecular Probes), a mitochondrial protein specific marker. All steps were carried out under reduced light conditions and solutions stored covered in foil to prevent bleaching. MitoTracker Green 1 mM stock was made from lyophilised powder and high-grade dimethyl sulfoxide (DMSO) and stored in the dark at -20 C in 20  $\mu$ l aliquots. COCs were treated with hyaluronidase as described in 3.2.1 to remove cumulus cells. Denuded oocytes were washed through fresh HEPES buffered  $\alpha$ MEM and then placed in a 40  $\mu$ l drop of 100 nM Mitotracker green (diluted in HEPES buffered  $\alpha$ MEM) under oil, in the dark for 15 mins at 37 C before being immediately loaded in confocal loading medium and imaged using confocal microscopy.

### **5.2.2.1 Analysis of oocyte mitochondrial distribution**

Images were assessed using Adobe Photoshop (version 6.0.1; Adobe Systems Inc., San Jose, USA) to calculate the mean pixel intensity of green fluorescence in different areas of the oocyte. This was performed as outlined in 5.2.1.1 (Figure 5.2). Each area of the oocyte assessed is expressed as a ratio of each other area to give a measure of distribution.



**Figure 5.2 Analysis of mitochondrial staining.**

Both JC-1 and MitoTracker Green were analysed by applying right-angled cross hairs over the image of the stained oocyte. Using Photoshop software, 3 boxes of equal pixel size were placed along each of the four arms of this axis in the peri-nuclear (A: light gray), intermediate (A: medium gray) and cortical (A: dark grey) regions respectively. Readings were then taken of pixel intensity in the appropriate spectrum (both red and green for JC1 and green for MitoTracker Green).

### **5.2.3 Mouse cumulus cell Microarray analysis**

Ribonuclease (RNase)-free conditions were maintained for surfaces, materials, chemicals and solutions and for handling of all materials used during RNA isolation. This included the use of disposable plasticware and disposable latex gloves which were replaced regularly during procedures and handling of materials.

#### **5.2.3.1 Cumulus cell collection for microarray**

To determine the effect of IVM oxygen concentration on cumulus cell gene expression, cumulus cells were collected following IVM at 2, 5, 10 and 20% oxygen concentration and from *in vivo* derived oocytes. COCs were collected and matured under varying oxygen environments or collected post-ovulation as previously described in 2.2.1-4. The presumptively mature complexes ( $\geq 20$  COCs) were transferred into 150  $\mu$ l drops of  $\alpha$ MEM HEPES 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 in 20  $\mu$ l of medium into a 1.5 ml tube, flash frozen in liquid nitrogen and stored at -80 C until use. To minimise changes in oxygen level, care was taken to ensure this procedure took only a few minutes.

#### **5.2.3.2 Cumulus cell RNA extraction**

Total RNA was isolated from cumulus cell samples using the RNeasy Micro Kit (Qiagen, Doncaster, USA). A maximum of 10 tubes were extracted at one time to prevent variation in extraction conditions. After each centrifugation step the flow through was discarded and the tube retained unless otherwise stated. All centrifugation steps were performed at room temperature. At thawing, 5  $\mu$ l of carrier RNA working solution (310  $\mu$ g supplied with kit, made up to 310  $\mu$ g/ml in 1 ml RNase-free water for storage at -20 C, diluted to 4 ng/ $\mu$ l working solution) was added to each sample. The volume of sample was adjusted to 300  $\mu$ l with addition of RLT buffer containing 10  $\mu$ l/ml  $\beta$ -mercaptoethanol and the solutions were mixed by vortex for 30

s. One volume (300  $\mu$ l) of 70% ethanol made with molecular grade 99.9% ethanol and RNase-free water was added to each sample and mixed by flicking the tube. The sample was then applied to the membrane surface of an RNeasy MinElute Spin Column in a 2 ml collection tube. The tube was closed gently and centrifuged at  $\geq 8000 \times g$  for 15 s. To wash the column, 350  $\mu$ l of buffer RW1 was added onto the membrane surface and the column centrifuged at  $\geq 8000 \times g$  for 15 s. DNA that may copurify was removed by addition of 80  $\mu$ l of DNase (1500 Kunitz units supplied with kit, final working concentration 0.34 Kunitz units/ $\mu$ l) to the membrane and incubation at room temperature for 15 minutes. To wash, 350  $\mu$ l of RW1 buffer was added to the column followed by centrifugation at  $\geq 8000 \times g$  for 15 s. A new collection tube was fitted to the column and 500  $\mu$ l of RPE buffer was added, the cap closed gently and the column centrifuged at  $\geq 8000 \times g$  for 15 s. To the surface of each column was added 500  $\mu$ l of 80% ethanol made with molecular grade 99.9% ethanol and RNase-free water. The lid was closed and the column centrifuged at  $\geq 8000 \times g$  for 2 min. Following this, the column was carefully transferred to a new 2 ml collection tube and centrifuged with the lid open at maximum speed for 5 minutes. The column was transferred to the final 1.5 ml collection tube and 14  $\mu$ l of RNase-free water was pipetted directly onto the centre of the column membrane and, with the lid closed, the column was centrifuged at maximum speed for 1 min. The elutant was stored at -80 C.

### **5.2.3.3 Affymetrix microarray**

A minimum of 4 extracted samples were pooled for each treatment and equal amounts of RNA from each pool (~100ng) were sent to the Australian Genome Research Facility (AGRF) in Melbourne, Australia. RNA quality and quantity was assessed by Agilent Bioanalyser (Agilent Technologies, Santa Clara, USA). The RNA then underwent Two-Cycle Target Labeling (Affymetrix, Santa Clara, USA) with biotin, followed by hybridisation to Affymetrix GeneChip Mouse Genome 430 2.0 GeneChip arrays, and scanning. These single channel microarrays (Affymetrix) contain > 45,000 probe sets to analyse the expression level of >



39,000 transcripts and variants, providing comprehensive coverage of the whole mouse genome. RNA integrity analysis, hybridisation and washing were performed by the AGRF facility according to the manufacturer's instructions. One replicate array was done for each experimental group – i.e. 5 in total.

#### **5.2.3.4 Microarray data analysis**

Due to the nature of the data, several different comparisons were able to be performed; *in vitro* vs *in vivo*, each oxygen treatment compared to *in vivo* and each oxygen treatment compared to one another.

Statistical methods to evaluate differential expression are not available for this study since there is  $n = 1$  per group. Two algorithms were therefore used to analyse the data, and probes common to both algorithms meeting a certain threshold (see below) for differential expression, were used to generate lists of differentially expressed probes for each comparison analysis.

For each chip, GCOS 1.4 software (Affymetrix) was used to generate CEL files. The MAS5.0 algorithm in GCOS 1.4 was used to scale the CEL files globally to a target intensity of 150 for generation of CHP files. These were then used for comparison analyses for each *in vitro* group minus the *in vivo* group (*in vivo* = baseline). Probes were sorted by detection, change, and signal log ratio (SLR); i.e. those "present", not "no change", and  $SLR \geq |1.0|$ . For the 2% and 5% analyses, due to the large differences in % probes present in the 2% and 5% oxygen chips compared to the *in vivo* group, probes were also selected if they were "absent" *in vitro*, and "present" *in vivo* (with the same change and SLR criteria above).

CEL file (raw) data were also analysed using the GCRMA algorithm implemented by Bioconductor ([www.bioconductor.org](http://www.bioconductor.org)) and the affyImGUI package. A linear model fit was used to compare separately the culture oxygen effects on the *in vivo* control cumulus cells – i.e. 20% O<sub>2</sub> minus *in vivo* control, 10% O<sub>2</sub> minus *in vivo* control, 5% O<sub>2</sub> minus *in vivo* control, and 2% O<sub>2</sub> minus *in vivo* control. Probes with M-values  $\geq |1.0|$  were selected. For each comparison, probes common to both lists generated by each algorithm, with

SLR- and M-values  $\geq |1.0|$  were used to generate lists of differentially expressed probes. For the *in vitro* vs *in vivo* analyses, and the 2, 5, 10 % oxygen group vs 20 % analyses, the final lists were submitted to a Venn diagram generator ([www.pangloss.com/seidel/Protocols/venn.cgi](http://www.pangloss.com/seidel/Protocols/venn.cgi)) to find overlaps between them.

## **5.3 Results**

### **5.3.1 Oocyte mitochondrial membrane potential**

To determine the effect of oxygen concentration during IVM on oocyte mitochondrial membrane potential, following IVM at 2, 5, 10 and 20% oxygen, oocytes were stripped of the surrounding cumulus cells and stained with JC-1 to allow analysis of both high and low polarised mitochondria. Mitochondrial membrane potential was examined over 3 experimental replicates (total number of oocytes examined per treatment  $\geq 26$ ). In all three areas examined (peri-nuclear, intermediate and cortical) and compared to all other oxygen treatments, IVM under 5% oxygen resulted in oocytes with significantly lower red:green pixel density which indicates less active mitochondria (graphed by area; Figure 5.3 and by treatment; Figure 5.4). In oocytes matured at 2, 10 and 20% oxygen the cortical region contains significantly more active mitochondria than the other two regions (peri-nuclear and intermediate). The cortical region of 5% oxygen IVM oocytes lacks this increase in highly polarised mitochondria (Figure 5.4).

### **5.3.2 Oocyte mitochondrial distribution**

To determine the effect of oxygen concentration during IVM on mitochondrial distribution, following IVM at 2, 5 or 20% oxygen or *in vivo* maturation, oocytes were stripped of the surrounding cumulus cells and stained with MitoTracker Green to facilitate analysis of the location of all mitochondria. Mitochondrial distribution was examined over 3 experimental replicates (total number of oocytes examined per group  $\geq 39$ ). The distribution of mitochondria to the intermediate area as a ratio of peri-nuclear distribution was not altered by oxygen treatment during IVM (Figure 5.5A). Following IVM at 5% oxygen, mitochondria distributed to the

cortical region relative to peri-nuclear distribution was significantly reduced compared to IVM at 20% (Figure 5.5B). Hence, relatively less mitochondria were distributed to the cortical region in oocytes matured at 5% oxygen. However, the ratio of cortical to peri-nuclear mitochondrial distribution was also lower in *in vivo* derived oocytes, when compared to oocytes matured at 2, 10 or 20% oxygen (Figure 5.5B).

### **5.3.3 Cumulus cell gene expression analysis**

To determine the effect of IVM oxygen concentration on cumulus cell gene expression, cumulus cells were collected following IVM at 2, 5, 10 and 20% oxygen concentration and from *in vivo* derived oocytes and the RNA from these cells extracted and processed for array hybridisation and scanning as previously described. A minimum of 4 replicates were pooled for each treatment with each replicate representing an equal proportion of the total RNA. These pools were used for cDNA synthesis, labelling, hybridisation and scanning. The data generated by this analysis should be considered preliminary only as array results with n=1 cannot be statistically tested.

#### **5.3.3.1 Microarray analysis of cumulus cells following maturation at differing oxygen *in vitro* vs *in vivo***

Data was analysed as reported above. The lists of differentially expressed probes for each separate *in vitro* oxygen group vs *in vivo* analysis are to be interpreted as those genes differentially expressed by cumulus cells following IVM culture at that oxygen concentration vs *in vivo*. Results are shown in Table 5.1 demonstrating large numbers of up- and down-regulated probes for each treatment.

There were 525 differentially expressed probes common to all 4 analyses (Figure 5.6) of which 377 were down regulated (Table 5.2) and 148 were up-regulated (Table 5.3). These can be interpreted as those genes differentially expressed by cumulus cells following *in vitro* maturation vs *in vivo*. Further to this, each treatment analysis can be taken separately, and interpreted as those genes differentially expressed by

cumulus cells following IVM culture at that oxygen concentration. Using this approach, there were 1316 genes differentially expressed by 2% (999↓, 317↑), 1541 (1286↓, 255↑) by 5%, 1676 (859↓, 817↑) by 10% and 1644 (843↓, 801↑) by 20% oxygen treated cumulus cells (Table 5.1).

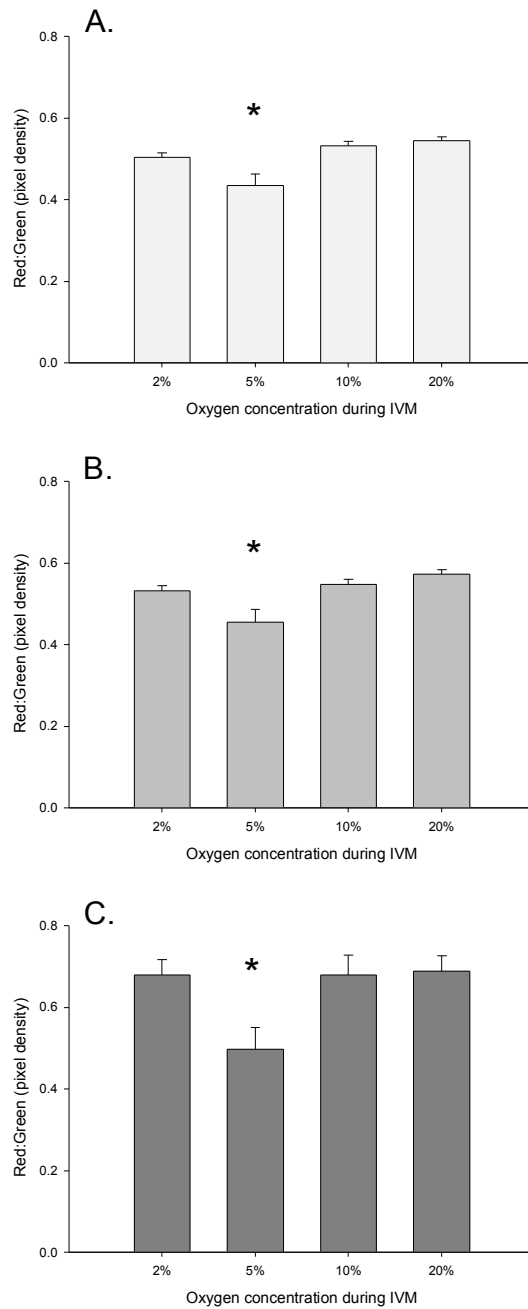
To extract biological meaning from these 525 probes, separate DOWN and UP lists of probes were submitted to DAVID 2007 (<http://david.abcc.ncifcrf.gov>, NIAID, NIH) to group genes into clusters based on functional similarity, and to highlight the most relevant annotation categories. However, due to the nature of this analysis and its reliance on annotation of the genes to identify functionality, we feel the results were largely incomplete. This type of analysis will be repeated in the future when more complete databases and improved software can be obtained.

### **5.3.3.2 Microarray analysis of cumulus cells following maturation in vitro at 2, 5 or 10% vs 20% oxygen**

To compare the effects of different oxygen concentrations on cumulus cell gene expression analyses were performed as in 5.3.3.1 except the 20% treatment was used as the 'baseline'. Results are shown in Table 5.4. Following IVM at 2, 5 and 10% oxygen, there were 273 (218↓, 55↑), 336 (305↓, 31↑) and 15 (15↑) cumulus cell probes respectively that were differentially expressed compared to 20% oxygen. Sample lists of genes down- and up-regulated following IVM at 2, 5, and 10% oxygen compared to 20% are given in Tables 5.5 and 5.6 [2%], Tables 5.7 and 5.8 [5%], and Table 5.9 [10%]. The Venn diagram (Figure 5.7) shows that there were 11 probes common to all 3 analyses, all of which were up-regulated. These may be interpreted as those probes differentially expressed following IVM culture under relatively low oxygen concentrations compared to atmospheric (20%) oxygen. In addition, at 2, 5 and 10% oxygen there were 218 (184↓, 34↑), 282 (271↓, 11↑) and 3 (3↑) differentially expressed genes that were exclusive to each oxygen treatment respectively. These can be interpreted as the genes that differ in expression in cumulus cells between each of these oxygen treatments to the 20% oxygen treatment.

### **5.3.3.3 Microarray analysis of cumulus cells following maturation in vitro at 5% vs. 2% oxygen**

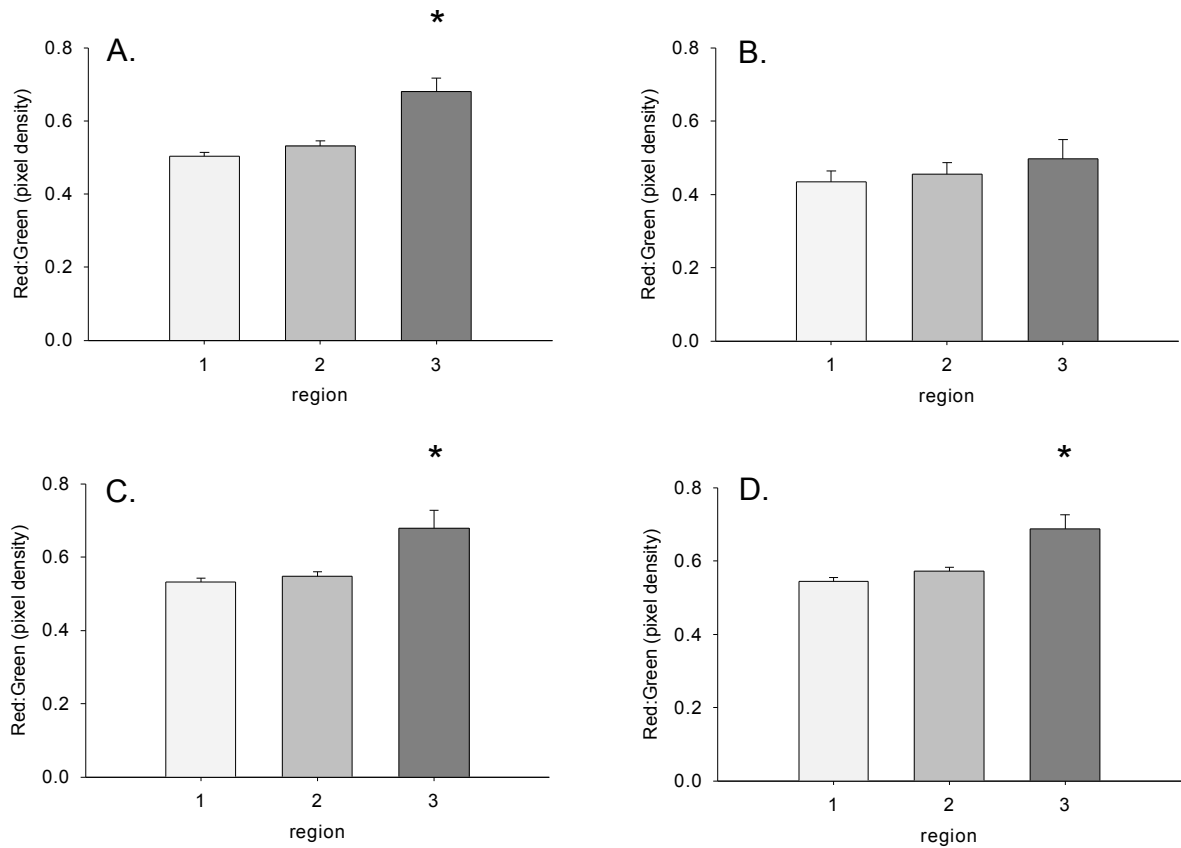
Analysis was also performed, as above, to compare cumulus cell gene expression at 5%, compared to 2% oxygen. For this analysis the 2% oxygen group was used as 'baseline'. There were 81 genes differentially expressed in cumulus cells exposed to 5% compared to 2% oxygen (Table 5.10) with 56 probes down regulated (Table 5.11) and 25 up-regulated (Table 5.12). Further information was extracted from the 5% O<sub>2</sub> and 2% O<sub>2</sub> analyses from the 4-analysis-Venn diagram (vitro vs vivo), and the 3-analysis-Venn diagram ([O<sub>2</sub>] vs 20%) to find overlapping probes exclusive to these comparisons (Table 5.13).



**Figure 5.3 Inner mitochondrial membrane potential ( $\Delta\psi_m$ ) in the peri-nuclear (A), intermediate (B) and cortical (C) regions of the oocyte as measured by the ratio of red (high polarisation) to green (low polarisation) fluorescence of JC-1 in oocytes matured under different oxygen concentrations.**

Data is expressed as mean  $\pm$  SEM. Mitochondrial membrane potential was examined over 3 experimental replicates with the total number of oocytes examined per treatment  $\geq$  26.

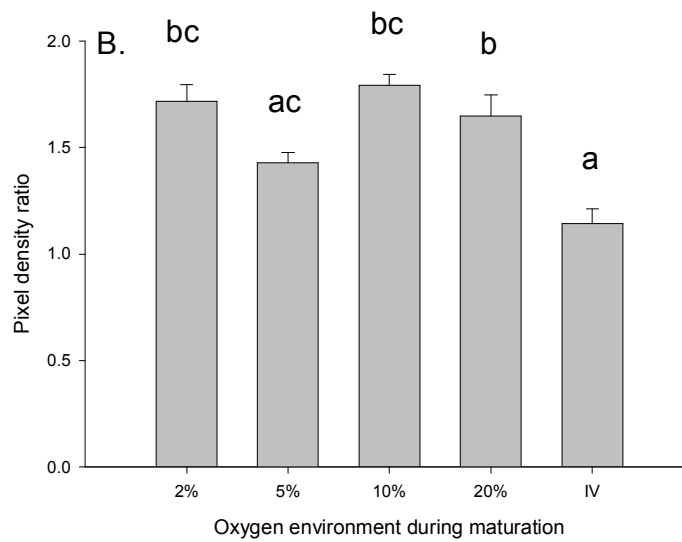
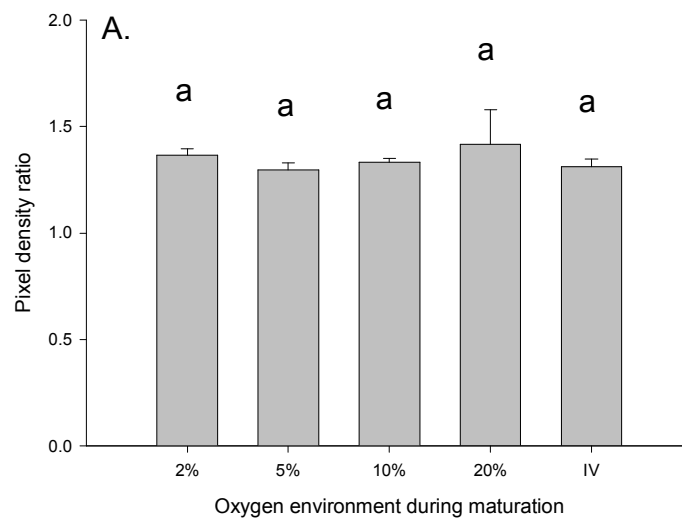
\* represents a significant difference from all other oocyte treatment groups.



**Figure 5.4** Inner mitochondrial membrane potential ( $\Delta\psi_m$ ) in oocytes matured under 2% (A), 5% (B), 10% (C) and 20% oxygen (D) across the 3 regions of the oocyte; peri-nuclear (1; light grey), intermediate (2; medium grey) and cortical (3; dark grey) as measured by the ratio of red (high polarisation) to green (low polarisation) fluorescence of JC-1.

Data is expressed as mean  $\pm$  SEM. Mitochondrial membrane potential was examined over 3 experimental replicates with a total number of oocytes examined per group  $\geq$  26.

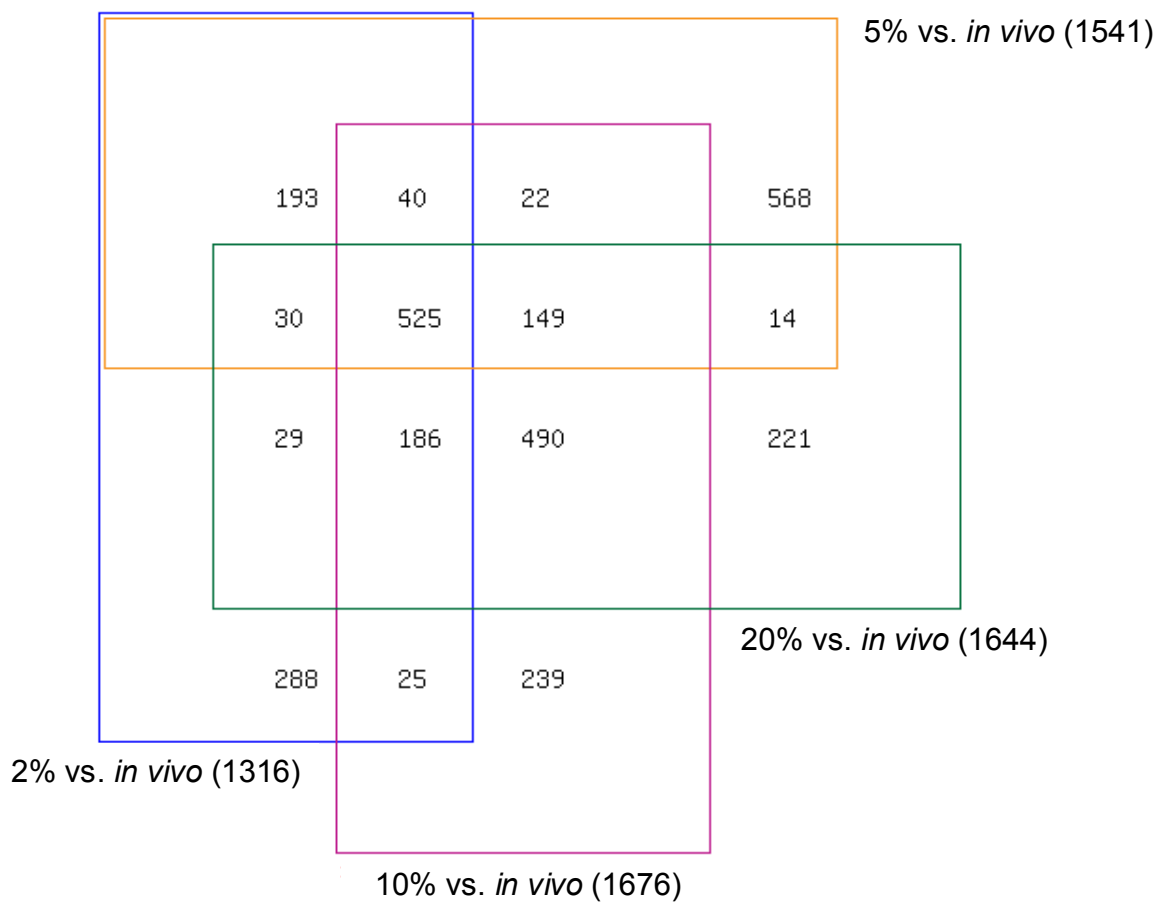
\* represents a significant difference from all other oocyte regions.



**Figure 5.5** Distribution of mitochondria to the intermediate (A) and cortical (B) regions in ratio to the peri-nuclear region in the oocyte following IVM under 2, 5, 10 and 20% oxygen or *in vivo* maturation (iv).

Data is expressed as mean  $\pm$  SEM. Mitochondrial distribution was examined over 3 experimental replicates with a total number of oocytes examined per group  $\geq$  39. Different superscripts denote a significant difference between treatment groups ( $P < 0.05$ ).





**Figure 5.6 Venn diagram for 4-way analysis of genes differentially expressed by cumulus cells following IVM culture under different oxygen concentrations compared to *in vivo* matured cumulus cells.**

The number of genes differentially expressed is presented above with each coloured rectangle representing an oxygen treatment vs. *in vivo* (2%; blue, 5%; orange, 10%; purple and 20%; green). Where rectangles overlap, the number within the overlapped area is the number of genes common to both lists (e.g. the number of unique genes differentially expressed by both 2 and 10% oxygen matured cumulus cells is 25). The number of genes up- or down-regulated compared to *in vivo* is given in Table 5.1.

**Table 5.1** The number of genes differentially expressed by cumulus cells following IVM culture under 2, 5, 10 and 20% oxygen compared to cumulus cells matured *in vivo*.

<b>Analysis</b>	<b>2% O<sub>2</sub> vs. <i>in vivo</i></b>	<b>5 % O<sub>2</sub> vs. <i>in vivo</i></b>	<b>10% O<sub>2</sub> vs. <i>in vivo</i></b>	<b>20% O<sub>2</sub> vs. <i>in vivo</i></b>
No. probes with $M \text{ \& } SLR \geq  1.0 $	1316: 999 ↓, 317 ↑	1541: 1286 ↓, 255 ↑	1676: 859 ↓, 817 ↑	1644: 843 ↓, 801 ↑
No. of these exclusive to this analysis	288: 243 ↓, 45 ↑	568: 516 ↓, 52 ↑	239: 100 ↓, 138 ↑	221: 94 ↓, 127 ↑

**Table 5.2 The top 10 (of 377) down-regulated genes in cumulus cells following *in vitro* culture (all 4 oxygen concentrations) vs. *in vivo*.**

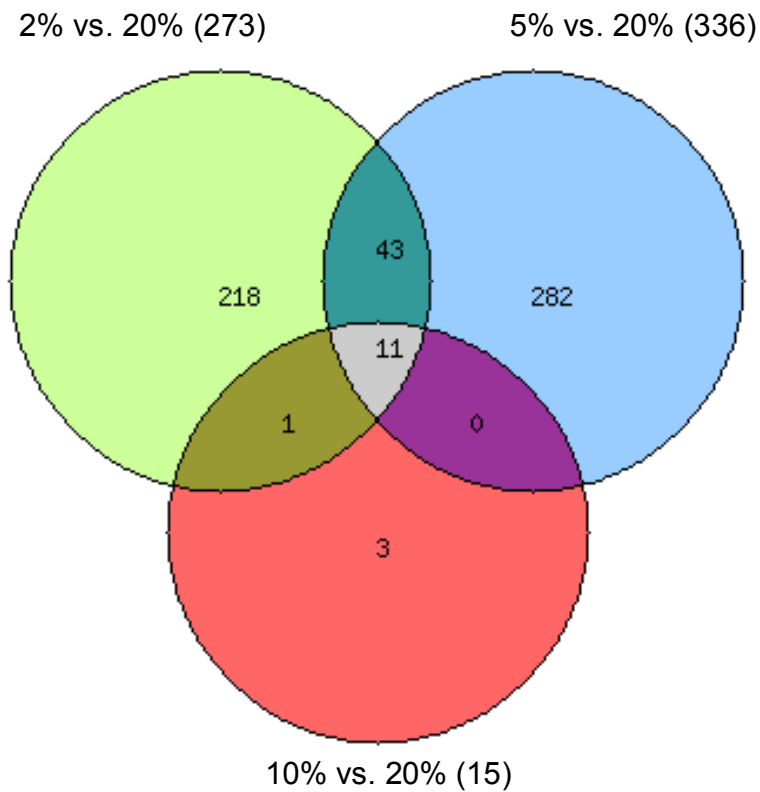
<b>Accession number</b>	<b>Gene code</b>	<b>Name</b>	<b>#Average fold change</b>
NM_008987.1	Ptx3	pentraxin related gene	94.9
AV309418	Ndrp1	n-myc downstream regulated gene 1	15.7
D67076.1	Adamts1	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif,1	24.6
AV315205	Alcam	activated leukocyte cell adhesion molecule	20.7
BB823350	Prkg2	protein kinase, cGMP-dependent, type II	24.2
BB550124	Tgm2	transglutaminase 2, C polypeptide	19.9
BB039510	Enpp3	ectonucleotide pyrophosphatase/phosphodiesterase 3	16.4
M94967.1	Ptgs2	prostaglandin-endoperoxide synthase 2	15.8
NM_134251.1	Slc12a8	solute carrier family 12 (potassium/chloride transporters), member 8	13.9
NM_133753.1	Errfi1	ERBB receptor feedback inhibitor 1	12.9

#The M values from the 2%, 5%, 10% and 20% oxygen treatment vs. *in vivo* analyses were averaged and used to calculate average fold change. The fold change may not reflect ranking position as the ranking of probes is based on both M and SLR values.

**Table 5.3 The top 10 (of 148) up-regulated genes in cumulus cells following *in vitro* culture (all 4 oxygen concentrations) vs. *in vivo*.**

<b>Accession number</b>	<b>Gene code</b>	<b>Name</b>	<b>#Average fold change</b>
BF225802	Igfbp5	insulin-like growth factor binding protein 5	64.4
NM_013706.1	Cd52	CD52 antigen	69.2
NM_007392.1	Acta2	actin. Alpha 2, smooth muscle, aorta	53.1
NM_013467.1	Aldh1a1	aldehyde dehydrogenase family 1, subfamily A1	30.2
AV026552	Hpgd	hydroxyprostaglandin dehydrogenase 15 (NAD)	19.0
NM_133859.1	Olfml3	olfactomedin-like 3	20.1
NM_009406.1	Tnni3	troponin 1, cardiac	19.4
AV259665	Amhr2	anti-Mullerian hormone type 2 receptor	17.6
BB453775	Mapk10	mitogen activated protein kinase 10	14.0
NM_008606.1	Mmp11	matrix metalloproteinase 11	16.0

#The M values from the 2%, 5%, 10% and 20% oxygen treatment vs. *in vivo* analyses were averaged and used to calculate average fold change. The fold change may not reflect ranking position as the ranking of probes is based on both M and SLR values.



**Figure 5.7 Venn diagram for 3-way analysis of genes differentially expressed by cumulus cells following IVM culture under different oxygen concentrations compared to IVM culture under 20% oxygen.**

The number of genes differentially expressed is presented above with each coloured circle representing an oxygen treatment (2%; green, 5%; blue and 10%; red). Where circles overlap, the number within the overlapped area is the number of genes common to both lists (e.g. the number of genes differentially expressed by both 2 and 10% oxygen matured cumulus cells, compared to 20% matured cumulus cells is 1). The number of genes up- or down-regulated compared to 20% is given in Table 5.4.

**Table 5.4** The number of genes differentially expressed by cumulus cells following IVM culture under 2, 5, and 10% oxygen compared to cumulus cells matured under 20% oxygen.

<b>Analysis</b>	<b>2% O<sub>2</sub> vs. 20% O<sub>2</sub></b>	<b>5% O<sub>2</sub> vs. 20% O<sub>2</sub></b>	<b>10% O<sub>2</sub> vs. 20% O<sub>2</sub></b>
No. probes with M & SLR ≥   1.0	273: 218 ↓, 55 ↑	336: 305 ↓, 31 ↑	15: 15 ↑
No. of these exclusive to this analysis	218: 184 ↓, 34 ↑	282: 271 ↓, 11 ↑	3: 3 ↑

**Table 5.5 The top 10 (of 218) down-regulated genes in cumulus cells matured *in vitro* under 2% oxygen vs. 20% oxygen.**

<b>Accession number</b>	<b>Gene code</b>	<b>Name</b>	<b>#Fold change</b>
NM_138656.1	Mvd	mevalonate (diphospho) decarboxylase	8.9
AF480860.1	Elovl6	ELOVL family member 6, elongation of long chain fatty acids (yeast)	8.4
D87867.1	Ugt1a6a	UDP glucuronosyltransferase 1 family, polypeptide A6A	6.6
NM_009003.1	Rab4a	RAB4A, member RAS oncogene family	6.3
AV216768	Phgdh	3-phosphoglycerate dehydrogenase	5.9
BC024423.1	Exosc1	exosome component 1	5.7
BM217803	Inpp5e	inositol polyphosphate-5-phosphatase E	5.7
BB350365	Ythdf1	YTH domain family 1	5.7
BI153391	Acsl4	acyl-CoA synthetase long-chain family member 4	5.6
BI453591	Gpd1l	glycerol-3-phosphate dehydrogenase 1-like	5.6

#Fold change was calculated using the M value only. The fold change may not reflect ranking position as the ranking of probes is based on both M and SLR values.

**Table 5.6 The top 10 (of 55) up-regulated genes in cumulus cells matured *in vitro* under 2% oxygen vs. 20% oxygen.**

<b>Accession number</b>	<b>Gene code</b>	<b>Name</b>	<b>#Fold change</b>
*BC024588.1	Kctd14	potassium channel tetramerisation domain containing 14	49.3 (28.3)
*AW552116	AI449310	expressed sequence AI449310	40.4 (28.9)
NM_009647.1	Ak3l1	adenylate kinase 3 alpha-like 1	14.1
AV309418	Ndrp1	N-myc downstream regulated gene 1	11.0
BB284358	Egln3	EGL nine homolog 3 (C. elegans)	8.8
NM_009760.1	Bnip3	BCL2/adenovirus E1B interacting protein 1, NIP3	8.7
AV305101	Pgk1	phosphoglycerate kinase 1	8.3
BC019856.1	Gulo	gulonolactone (L-) oxidase	7.6
AI606078	Vegfa	vascular endothelial growth factor A	7.3
BB748743	NA	NA (probe 1447352_at)	7.2

\*Denotes genes appearing more than once in the top 10 (i.e. more than one probe). These are only listed once in the list place corresponding to the most significant occurrence. The fold change of subsequent occurrences appears in brackets. Probes with different accession numbers were not combined in this way. #Fold change was calculated using the M value only. The fold change may not reflect ranking position as the ranking of probes is based on both M and SLR values.



**Table 5.7 The top 10 (of 305) down-regulated genes from cumulus cells matured *in vitro* under 5% oxygen vs. 20% oxygen.**

<b>Accession number</b>	<b>Gene code</b>	<b>Name</b>	<b>#Fold change</b>
D87867.1	Ugt1a6a	UDP glucuronosyltransferase 1 family, polypeptide A6A	12.8
AW208417	Plaa	phospholipase A2, activating protein	8.7
BG067039	Amot	angiomin	8.7
BM240684	AI848100	expressed sequence AI848100	7.8
BI153391	Acsl4	acyl-CoA synthetase long-chain family member 4	7.8
AA673371	4933439C20Rik	RIKEN cDNA 4933439C20 gene	7.5
AV230488	Klf3	Kruppel-like factor (basic)	7.2
AI323642	Tcf7	transcription factor 7, T-cell specific	7.0
BC026131.1	Slc7a5	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	6.9
NM_025292.1	Synj2bp	synaptojanin 2 binding protein	6.8

#Fold change was calculated using the M value only. The fold change may not reflect ranking position as the ranking of probes is based on both M and SLR values.

**Table 5.8 The top 10 (of 31) up-regulated genes from cumulus cells matured *in vitro* under 5% oxygen vs. 20% oxygen.**

<b>Accession number</b>	<b>Gene code</b>	<b>Name</b>	<b>#Fold change</b>
*BC024588.1	Kctd14	potassium channel tetramerisation domain containing 14	30.8 (22.6)
*AW552116	AI449310	expressed sequence AI449310	22.5 (17.7)
BB357165	Gcnt2	glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	20.0
BB129990	NA	NA (probe 1456464_x_at)	10.5
*NM_023119.1	Eno1	enolase 1, alpha non-neuron	7.3 (6.7)
BC004017.1	Eno1	enolase 1, alpha non-neuron	5.9
NM_008828.1	Pgk1	phosphoglycerate kinase 1	5.6
BB240086	BB220380	expressed sequence BB220380	5.6
NM_010699.1	Ldha	lactate dehydrogenase A	5.3
NM_009647.1	Ak3l1	adenylate kinase 3 alpha-like 1	5.3

\*Denotes genes appearing more than once in the top 10 (i.e. more than one probe). These are only listed once in the list place corresponding to the most significant occurrence. The fold change of subsequent occurrences appears in brackets. Probes with different accession numbers were not combined in this way. #Fold change was calculated using the M value only. The fold change may not reflect ranking position as the ranking of probes is based on both M and SLR values.

**Table 5.9 The top 10 (of 15) up-regulated genes from cumulus cells matured *in vitro* under 10% oxygen vs. 20% oxygen**

<b>Accession number</b>	<b>Gene code</b>	<b>Name</b>	<b>#Fold change</b>
NM_030696.1	Slc16a3	solute carrier family 16 (monocarboxylic acid transporters), member 3	5.1
*NM_009647.1	Ak3l1	adenylate kinase 3 alpha-like 1	4.5 (3.8)
BC024588.1	Kctd14	potassium channel tetramerisation domain containing 14	3.3
*AW552116	AI449310	expressed sequence AI449310	2.8 (2.7)
*NM_023119.1	Eno1	enolase 1, alpha non-neuron	2.8 (2.3)
BC027196.1	Pdk1	pyruvate dehydrogenase kinase, isoenzyme 1	2.3
AK018763.1	Agt	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	2.3
NM_010699.1	Ldha	lactate dehydrogenase A	2.3
NM_008828.1	Pgk1	phosphoglycerate kinase 1	2.2
BC004017.1	Eno1	enolase 1, alpha non-neuron	2.2

\*Denotes genes appearing more than once in the top 10 (i.e. more than one probe). These are only listed once in the list place corresponding to the most significant occurrence. The fold change of subsequent occurrences appears in brackets. Probes with different accession numbers were not combined in this way. #Fold change was calculated using the M value only. The fold change may not reflect ranking position as the ranking of probes is based on both M and SLR values.

**Table 5.10** The number of genes differentially expressed by cumulus cells following IVM culture under 5% oxygen compared to cumulus cells matured under 2% oxygen.

Analysis	5% O <sub>2</sub> vs. 2% O <sub>2</sub>
No. probes with M & SLR $\geq$   1.0	81: 56 ↓, 25 ↑

**Table 5.11 The top 10 (of 56) down-regulated genes from cumulus cells matured *in vitro* under 5% oxygen vs. 2% oxygen**

<b>Accession number</b>	<b>Gene code</b>	<b>Name</b>	<b>#Fold change</b>
BC004724.1	Fn1	fibronectin 1	4.5
AV309418	Ndrp1	N-myc downstream regulated gene 1	4.4
BB471300	6820431F20Rik	RIKEN cDNA 6820431F20 gene	3.5
BC019131.1	1110067D22Rik	RIKEN cDNA 1110067D22 gene	3.4
AV026552	Hpgd	hydroxyprostaglandin dehydrogenase 15 (NAD)	3.4
AV021813	Auts2	autism susceptibility candidate 2	3.4
AW536432	Hs6st2	heparin sulphate 6-O-sulfotransferase 2	3.2
BB748743	NA	NA (probe 1447352_at)	3.1
NM_011825.1	Grem2	Gremlin 2 homolog, cysteine knot sperfamily ( <i>Xenopus laevis</i> )	3.1
AI255184	Evi5	ecotropic viral integration site 5	3.1

#Fold change was calculated using the M value only. The fold change may not reflect ranking position as the ranking of probes is based on both M and SLR values.

**Table 5.12 The top 10 (of 25) up-regulated genes from cumulus cells matured *in vitro* under 5% oxygen vs. 2% oxygen**

<b>Accession number</b>	<b>Gene code</b>	<b>Name</b>	<b>#Fold change</b>
AV074978	NA	NA (probe 14444616_x_at)	4.2
BG073047	Tpx2	TPX, microtubule-associated protein homolog ( <i>Xenopus laevis</i> )	3.8
BB357165	Gcnt2	glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	3.8
BB317923	Coro2b	coronin, actin binding protein, 2B	3.3
BB207248	Pik3cd	phosphatidylinositol 3-kinase catalytic delta polypeptide	3.2
AK013970.1	Gmfb	glia maturation factor, beta	3.1
AV007213	Zfp383	zinc finger protein 383	2.9
BI247584	Fdps	farnesyl diphosphate synthetase	2.8
BB286270	E2f5	EF2 transcription factor 5	2.5
BB245119	Uck2	uridine-cytidine kinase 2	2.4

#Fold change was calculated using the M value only. The fold change may not reflect ranking position as the ranking of probes is based on both M and SLR values.

**Table 5.13** The number of genes differentially expressed by cumulus cells following IVM culture under 2 and 5% oxygen compared to cumulus cells matured *in vivo* or at 20% oxygen.

<b>Analyses</b>	<b>2% O<sub>2</sub> vs. <i>in vivo</i> &amp; 5% O<sub>2</sub> vs. <i>in vivo</i></b>	<b>2% O<sub>2</sub> vs. 20% O<sub>2</sub> &amp; 5% O<sub>2</sub> vs. 20% O<sub>2</sub></b>
No. exclusively overlapping probes with M & SLR ≥   1.0	193: 185 ↓, 7 ↑	43: 34 ↓, 9 ↑

## 5.4 Discussion

The process of cytoplasmic maturation is a complex process loosely defined as the gaining of factors and cellular machinery which enable proper development once fertilised. The activity and distribution of mitochondria form a part of this process with changes occurring through maturation and early development (Bavister and Squirrell 2000; Sun *et al.* 2001). The changes are necessary and conducive to proper development and disruption in distribution and function of the mitochondria in the oocyte will impact on the subsequent competence of that oocyte .

The mitochondria are responsible for the generation of energy in the oocyte and as this process requires oxygen, changing the amount of available oxygen may impact on the efficiency of energy production. The results of this study show that the oxygen environment during maturation *in vitro* is able to alter both the activity and distribution of mitochondria, with oocytes matured under 5% oxygen exhibiting different mitochondrial patterning compared to the other oxygen concentrations used. The 5% oocytes did not exhibit the 'hyper-polarised ring' seen in the other oxygen treatment groups and which has been previously shown by Van Blerkom and colleagues (2002; 2003) to be a characteristic of mouse oocytes. Furthermore, the 5% oxygen matured oocytes also had less mitochondria located in this outer region. However, this is comparable to the distribution in the *in vivo* matured oocytes.

Our data is also partially supported by recent findings from Van Blerkom, Davis & Thalhammer (2008) in that extrinsic factors such as the oxygen concentration during IVM can alter mitochondrial polarity within the oocyte. Nevertheless, we have not investigated any other extrinsic or intrinsic factors, such as nitric oxide production as is the case within the study by Van Blerkom, Davis & Thalhammer (2008).

Interestingly, it has been suggested that highly active mitochondria involved in the generation of ATP undergo constant dissipation of the membrane potential and thus should have a moderate potential reading



instead of a high one (Diaz *et al.* 1999). In mouse oocytes, it has also been shown that enough membrane potential exists even under conditions of reduced ATP synthesis (caused by reversal of ATP synthase activity) to allow the J-aggregates to form (Van Blerkom *et al.* 2003). With these suggestions in mind, the results of this study still show a difference in mitochondrial behaviour under varying oxygen concentrations. Furthermore, this also suggests the impact on ATP synthesis could potentially be altered to a different extent than evidenced by JC1 staining and requires further investigation by measurement of ATP levels.

In Chapter 4, it was shown that IVM under 5% oxygen resulted in a lighter conceptus following IVF, IVC and embryo transfer. It was this same treatment group that exhibited reduced mitochondrial activity and distribution in the oocyte. These results suggest a potential link between altered mitochondrial parameters and the subsequent development of the resulting conceptus.

As has been mentioned previously, a close relationship exists between the cumulus cells and oocyte. This relationship may be a potential mechanism by which effects on oocyte quality and subsequent development are mediated. Therefore it is important that the study of IVM environment (in this case, oxygen concentration) also consider the impact on cumulus cells. Defining and developing markers of oocyte quality is the aim of much current oocyte biology research. Traditional parameters used to predict oocyte quality involve morphological analysis of the COC including measures of expansion of the cumulus oophorus and characteristics of the cytoplasm, first polar body and the meiotic spindle (Wang and Sun 2007). These types of morphological scoring systems come under considerable scrutiny, perhaps understandably so, due to the subjective nature of visual indicators. Other parameters which have no 'operator biases' such as molecular predictors show promise. However, these types of measures also have associated pitfalls. Any measure must be performed without destruction of the oocyte or disruption of any processes required for developmental competence. Therefore, one approach often studied is the measurement of the health of the oocyte by the health of the associated cumulus cells. The theory being that the communication between the

surrounding somatic cells and the oocyte determines oocyte competency (Gandolfi *et al.* 2005). These cells are often removed during IVF cycles and discarded, offering a unique opportunity for researchers and clinicians alike to gain valuable information.

The relationship between the expression of some genes in cumulus cells and developmental competence has been established. In human cumulus cells, the expression of *GREM1* was found to predict the developmental competency as measured by maturity, fertilisation potential and embryo quality (McKenzie *et al.* 2004). Likewise, through microarray of human cumulus cells, the abundance of *PTX3* was discovered to correlate with developmental competence (Zhang *et al.* 2005). Not surprisingly, in this study, *Ptx3* was found to be down regulated in cumulus from IVM COCs compared to *in vivo* derived cumulus. This correlates with the known decreased developmental potential of IVM oocytes.

Gremlin 2 (also known as *prdc*) and its paralog Gremlin have been shown to be antagonists of the BMPs (Sudo *et al.* 2004; Im *et al.* 2007). Gremlin 2 expression in granulosa cells suggests its role in follicular development could be to antagonise the theca-derived BMPs, ensuring their regulation (Sudo *et al.* 2004). *Grem2* was found to be down regulated in the cumulus cells of COCs matured under 5% oxygen, when compared to their 2% oxygen counterparts. If *Grem2* is a marker of cumulus cell function, like *Grem1*, this would suggest that oocytes matured under 5% oxygen have a lower developmental potential than those matured under 2% oxygen. However, as the role of *Grem2* in cumulus cells is not characterised, this is currently speculation. Additionally, differences in gene expression with lower fold changes such as this do need to be verified by real-time PCR.

*Adamts1* (a disintegrin and metalloproteinase with thrombospondin-like repeats) was found to be down regulated in the IVM derived cumulus compared to the *in vivo* derived. *Adamts1* and its family members act to cleave matrix proteins such as Versican in the expanding COC and are believed to play a role in the process of ovulation (Richards 2005). *Adamts1* deficient COCs show decreased rates of fertilisation (Brown

et al, personal communication) suggesting the down regulation in IVM cumulus cells could potentially negatively impact the developmental competence of the associated oocytes.

Genes that were up-regulated following IVM compared to *in vivo* include the insulin-like growth factor binding protein 5 (*Igfbp5*). Some of the IGF binding proteins (including -5) are known to be associated with the extracellular matrix (Clemmons 1998) where they have been shown to be regulated by FSH in several species (Adashi *et al.* 1990; Mondschein *et al.* 1990). It has been suggested that FSH regulates the ability of the extracellular matrix to 'entrap' these proteins once secreted and therefore provides a mechanism for differential regulation of IGF action in the COC (Ingman *et al.* 2000). The up-regulation of *Igfbp5* in cumulus cells of COC matured *in vitro* may be due to the presence of FSH in the maturation medium or perhaps as a response to the lack of IGFs in the *in vitro* system.

Likewise, the up-regulation of *Amhr2* in the *in vitro* matured cumulus cells could be caused by the removal of follicular signals. Anti-mullerian hormone (AMH) is a member of the TGF $\beta$  superfamily of growth and differentiation factors (Grujters *et al.* 2003). It is expressed in the granulosa cells of pre-antral and small antral follicles (reviewed by Durlinger *et al.* 2002) and as such can be used as a measurement of the remaining ovarian pool (Grujters *et al.* 2003). The receptor co-localises with the hormone (Baarends *et al.* 1995) which becomes restricted to the cumulus cells following antrum formation (Salmon *et al.* 2004).

Of the 525 genes changed *in vitro* compared to *in vivo*, the oxygen treatment during IVM which had the highest number of unique genes changed in the cumulus cells following IVM was 5% oxygen (Table 5.1), suggesting that these cells were most different to those matured *in vivo*. It is also interesting to note that the majority of change involved the down-regulation of genes.

The effects of varying oxygen concentration also influenced cumulus cell gene expression. Oxygen regulated gene expression is well described in somatic cells (reviewed by Semenza 1998). The HIFs are

transcription factors known to mediate the regulation of a number of genes in response to a reduced oxygen environment (Semenza 1998). These oxygen responsive genes (or HIF target genes) contain hypoxia response elements (HREs) to which the HIF transcription complex can bind and induce transcription (for a recent review see Kenneth and Rocha 2008). HIFs are the main players in the physiological response to restore and maintain homeostasis under hypoxic conditions (Semenza 1998). Not surprisingly, HIF target genes are largely involved in the logical short term responses to these conditions such as erythropoiesis and glycolysis and if the hypoxic condition is persistent, the longer term solution of angiogenesis (Semenza 1998).

The HIF responsive genes *Eno1*, *Ldha*, *Ak3l1* and *Pgk1* were all up-regulated in cumulus from IVM at 2, 5 and 10% compared to 20% oxygen. All of these genes are involved in glucose metabolism suggesting these cells were initiating the HIF response to decreased levels of oxygen. The response also seems to be graded with some genes such as *Pgk1* having a more dramatic change as oxygen decreased (at 2, 5 and 10% there was 8.3, 5.6 and a 2.2 fold change respectively compared to 20% oxygen). In addition, two other genes were also consistently up-regulated in these groups; *Kctd14* and *A1449310*. *Kctd14* is a potassium channel tetramerisation domain. These tetramerisation domains are involved in the formation of the potassium channel pore structure (Bixby *et al.* 1999). *A1449310* is an expressed sequence tag of which the function is unknown. The regulation of these two genes in cumulus cells and why they are so consistently and dramatically up-regulated under these conditions is unknown.

Following IVM at 2 and 5% oxygen, in addition to those HIF responsive genes already mentioned, we find that there are several other HIF responsive genes commonly up-regulated in these cumulus cells compared to 20% oxygen IVM cumulus cells. *Vegfa*, a well known and characterised HIF target gene is involved in angiogenesis under hypoxic conditions and is up-regulated under these conditions. *Bnip3* is also up-regulated (although it is not shown in the 'top 10' list given for 5% but appears further down the list). The

Nip3 protein is a member of the Bcl-2 family, a well known pro-apoptotic factor induced under hypoxic conditions (Bruick 2000). This up-regulation of a pro-apoptotic gene may have implications for the health of cumulus cells under these oxygen conditions; however this was not investigated here. *Ndr1* is also up-regulated in cumulus cells under these conditions. This gene is a member of the N-myc downregulated gene family and encodes for a cytoplasmic protein involved in stress responses, hormone responses, cell growth and differentiation (Ellen *et al.* 2008). *Ndr1* mRNA while ubiquitous in many tissues, has been found to be strongly up-regulated under hypoxic conditions, in differentiating cells and under many other cellular stresses, but is down-regulated under conditions of cell growth (Kokame *et al.* 1996; van Belzen *et al.* 1997; Kurdistani *et al.* 1998; Zhou *et al.* 1998). The up-regulation seen here therefore suggests that the cumulus cells at 2 and 5% oxygen are under stress.

*Elg1* (*Phd3*) was up-regulated in cumulus cells from 2% IVM COCs. *Elg1* encodes for prolyl hydroxylase domain proteins (PHDs) that hydroxylate the  $\alpha$ -subunits of HIF-1 and -2 (usually under normoxia) which signal their polyubiquitination and proteasomal degradation and so regulate HIF abundance (Ivan *et al.* 2001; Jaakkola *et al.* 2001; Yu *et al.* 2001). Counter intuitively, some isoforms (PHD2 and 3) are also transiently up-regulated under hypoxic conditions (Berra *et al.* 2003; Marxsen *et al.* 2004; Rantanen *et al.* 2008).

The up-regulation of *Phd3* in these cumulus cells provides evidence that these cells are experiencing hypoxic conditions and validates the use of this model for inducing a low oxygen environment. Overall, use of this particular experimental model is ratified by the evident increase in the HIF mediated response as oxygen level during IVM decreased. While the mounting of the HIF response at low levels of oxygen was to be expected, the upregulation of some HIF responsive genes at 10% oxygen levels is intriguing. However, to determine if 10% oxygen is seen as a hypoxic environment by the cumulus cells would require further investigation. It is possible that at this level of oxygen these genes were upregulated by some other

mechanism other than the typical HIF pathway characterised by the stabilisation, dimerisation, and binding of the HIF-1 complex.

There is evidence in the literature to suggest that HIF-2 $\alpha$  (HIF-2 $\alpha$  being the oxygen-sensitive subunit in the HIF-2 dimer rather than HIF-1 $\alpha$ ) does not require the the same degree of hypoxia for stabilisation (Wiesener *et al.* 1998; Wiesener *et al.* 2003; Holmquist-Mengelbier *et al.* 2006). A greater increase in HIF-2 $\alpha$  than HIF-1 $\alpha$  protein was observed under exposure to 'mild hypoxia' (5% oxygen) in a range of human cell lines (Wiesener *et al.* 1998). It is possible that the HIF type responses seen in this study at 5 and 10% oxygen levels could be mediated by this alternative pathway. As there are many common target genes of HIF-1 and -2, it is difficult to ascertain if HIF-1 or -2 is the predominant transcription factor from our current data. Further to this, the subunits themselves are regulated mainly at protein level (by increased protein stability, see review by Semenza 1998) rather than by mRNA abundance and so additional investigations would be required to determine if HIF-2 was responsible for eliciting the up-regulation of HIF target genes.

Of the genes down-regulated across treatment groups compared to 20% oxygen IVM cumulus cells, a common gene function emerged. Following IVM at 2 and 5% oxygen, cumulus cells were found to have a number of genes down-regulated that play a role in either fatty-acid metabolism (such as *Acs14* and *Elovl6*) or cholesterol synthesis (*Sqle* and *Phgdh* in both 2 and 5% and *Fdps* under 2% oxygen exclusively). For most of these genes it is not known if they are specifically regulated by oxygen [*Sqle* has been shown to be REDOX regulated (Li and Porter 2007)] but this data suggests that these genes were more highly expressed under 20% oxygen and are perhaps reduction-oxidation equilibrium sensitive. A down regulation of genes involved in cholesterol synthesis may have implications for steroid production by the cumulus cells.

When looking at the number of genes changed by oxygen treatment during IVM, it is perhaps not surprising to see that the gene expression profiles of the 10% and 20% oxygen treated cumulus cells are not very different, with only 3 exclusive genes differing (Table 5.4, Figure 5.7). However, compared to 20%, the 5%

oxygen treatment during IVM results in the greatest number of unique genes (282) being altered (Table 5.4, Figure 5.7), meaning these two groups are the most different in their expression. The 2% IVM treatment also differs considerably to the 20% with 218 unique genes being changed (Table 5.4, Figure 5.7). The comparison of cumulus cell gene expression following IVM at 5% oxygen with 2% shows us that these two groups also differ from each other in the expression of 81 genes (Table 5.10). In previous chapters we have seen non-linear effects on development following IVM at these (2 and 5%) oxygen concentrations. The 2% treatment elicits a response at the blastocyst stage in the form of increased cell number (Chapter 3), whereas the 5% treatment has an effect post-implantation resulting in decreased fetal and placental weight (Chapter 4). It is clear from the results of this study that the effect of oxygen concentration during IVM on oocyte metabolism and on the cumulus cell gene expression profile is also non-linear. We would suggest that the impact of unique IVM oxygen concentrations on particular stages of further development must therefore be mediated through unique mechanisms.

It is pertinent at this point to reiterate that the micro array data presented here is of a single replicate array and firm conclusions cannot be drawn. However, the experiment has been useful in highlighting several genes of interest, including those known to be oxygen responsive. This provides a basis for future research where the relative levels of these genes can be confirmed by further independent array replicates or by quantitative PCR.

These results were also gained using one particular IVM system. If these studies were to be repeated using a different IVM system, different results may be generated. It is important to take this into consideration when comparing results across studies. The results from this study show that the impact of IVM oxygen concentration is complex. Because of this complexity, care should be taken in the study of oxygen effects in this way. Design of experiments to test the impact of oxygen in IVM systems should cover a full range of oxygen concentrations to limit the risk of missing potentially important, but unexpected, interactions.

## **6 General discussion**



## 6.1 Introduction

There is no doubt that the quality of the oocyte plays a large role in determining outcomes once fertilised. This is most certainly true regardless of whether the oocyte completes development *in vivo* or *in vitro*. The increased use of oocyte maturation *in vitro* both in the clinical setting and in the lab drives the need for an increased understanding of the potential the maturation environment has for impacting oocyte quality.

While the embryo culture environment has been well studied, the conditions for oocyte maturation *in vitro* have not yet been optimised. Furthermore, our understanding of the follicle environment during oocyte maturation *in vivo* and how this impacts on oocyte quality is far from complete. The oxygen concentration is an aspect of the maturation environment which has not been adequately studied, although we know this is one factor that does vary *in vivo*. The present study investigated the direct effects of a wide range of oxygen levels during oocyte maturation *in vitro* on outcome parameters measured across development including in the COC, subsequent embryo and conceptus. This is the first time such an examination of outcome measures had been conducted on such a wide range of oxygen concentrations. Previous studies have been limited in the use of oxygen levels considered to be 'low' as well as conservative in the study of outcomes. The results from this study provide evidence that the oxygen level present during IVM has significant effects on parameters measured at all stages of development, but that the response is not consistent along the developmental continuum. In this chapter, the response during development to the IVM oxygen concentration is discussed and the future research required to further understanding in this area is highlighted.

## **6.2 The impact of oxygen concentration during mouse IVM on measures of developmental competence**

Historical studies looking at the production of a polar body by the oocyte during mouse oocyte maturation *in vitro* have led to the thought that a 5% oxygen environment is optimal for this process (Haidri *et al.* 1971; Gwatkin and Haidri 1974). Furthermore, oxygen levels below 5% have been regarded as deleterious to oocyte maturation (Haidri *et al.* 1971; Boland *et al.* 1994) and so were excluded from most modern studies of IVM oxygen conditions. The experiments in Chapter 3 of this thesis demonstrate that (under the conditions used) IVM oxygen environment causes no measurable differences in the ability of oocytes to reach the MII stage of nuclear maturation, fertilise, cleave or reach the blastocyst stage of embryo development, disagreeing with these historical findings. There are many possible reasons for this difference. The work by Hashimoto *et al.* (2000a) clearly demonstrates that altering IVM media components (in this case, increasing glucose concentration) can dramatically improve results following IVM at low oxygen. Other changes to IVM systems that have the potential to be responsible for this discrepancy could include; changes in the COC/volume of medium ratio thus altering the availability of substrates, the type of culture vessel used (dishes, tubes or plates) which could alter the dynamics of the system, the use of gonadotrophin in the culture system, the timing of maturation and fertilisation and the use of different genetic strains to name a few. Changes to IVM systems made since these initial studies, (for example, developments in quality control practices and handling techniques) are sure to have improved outcomes over time.

Nevertheless it is clear that when using the IVM systems available today, this historically-based dogma is not a valid reason to discount the use of any oxygen levels, including levels <5% oxygen in studies of IVM oxygen environments. Instead, oxygen levels below 5% should be considered for inclusion in the study design to ensure an accurate picture of the response to oxygen in that system is gained. Likewise, in these

'improved' systems where usual outcome measures are virtually unaltered, it may be advisable to include other measures that look past the ability of an oocyte to 'develop to blastocyst' as an indicator of developmental competence.

As seen in Chapter 3, while the oxygen environment during IVM does not affect the rate of blastocyst development it does impact on the cellular development of resulting blastocysts, with total blastocyst cell number increasing as oxygen concentration during oocyte maturation decreases. This change in cell number can be attributed almost exclusively to a change in the number of trophectoderm cells. As stated in Chapter 1, blastocysts derived from mouse oocytes matured at 5% oxygen have previously been found to have significantly higher cell number compared to those matured at 20%; however, the cellular compartment responsible for this change was not identified (Preis *et al.* 2007). A reduced oxygen concentration during embryo culture is thought to increase blastocyst cell numbers through the reduction of reactive oxygen species (ROS) production (Orsi and Leese 2001). A reduced oxygen concentration during IVM would also limit production of ROS and it seems this also flows onto the resulting blastocyst cell number.

Also in Chapter 3, TUNEL identified only a small proportion of apoptotic cells in the blastocysts derived from oocytes matured at 2% oxygen, suggesting the majority of the additional cells were viable. The immunohistochemistry performed with markers for TE and ICM (Cdx2 and Oct4 respectively), reported in Chapter 3, elucidated that morulae derived from oocytes matured at 2% oxygen had an increased number of cells expressing both markers, indicating the fate of these cells to be not yet fully determined at this time. In light of the increased number of TE cells in the blastocyst following IVM at 2%, it is likely that these partially determined cells went on to form true trophectoderm cells, although further investigation of the developing blastocysts is needed to confirm this.

The potential effects of oxygen treatment-induced changes to blastocyst cellular composition on implantation and development following transfer were examined in Chapter 4. It was hypothesised that altered cellular make-up of the blastocyst would impact on further development of the embryo and conceptus. At the blastocyst stage, trophoctoderm cell numbers were altered. As trophoctoderm is the cellular compartment of the blastocyst that eventually forms the fetal-maternal interface within the placenta it was expected that differences in cellular composition of the blastocyst may be reflected by effects on implantation and/or placentation. This was not found to be so. There was no difference in implantation and survival to Day 18 regardless of the oxygen concentration used during IVM. However, following IVM at 5% oxygen, the weight of the subsequent conceptus was found to be reduced. No comment can be made on the morphological parameters of the placenta as placental structure was not investigated. However, there was no change in the fetal/placental weight ratio suggesting placental efficiency was appropriate.

From these studies, no clear linear correlation was found between oxygen concentration during IVM and subsequent effects on development. Instead, unique effects at different stages of development were observed following IVM at the lower oxygen concentration (2 and 5% oxygen). This suggests a complex interplay between several systems may be responsible for these different outcomes. Evidence was found in this study which may allude to these potential mechanisms and this is discussed here.

### **6.3 Potential oxygen dependent pathways influencing outcomes**

In Chapter 5 the oxygen environment during maturation *in vitro* was shown to alter both the activity and distribution of mitochondria in the oocyte, specifically following IVM in 5% oxygen. Oocytes matured in a 5% oxygen environment also exhibited differences in fetal and placental weight following embryo transfer as shown in Chapter 4. It is possible that the metabolic impact of a 5% oxygen environment on the oocyte, prior to both fertilisation and embryo development is capable of programming differences in the conceptus. The changes in these mitochondrial parameters seen following maturation at 5% oxygen indicate change in

the metabolism, and therefore energy production, of the oocyte specifically caused by the 5% oxygen environment. Perhaps it is this change in energy supply that is responsible for the programming of the conceptus.

Further to this, array studies of the cumulus cells following IVM at various oxygen concentrations (Chapter 5) highlighted the “uniqueness” of the 5% oxygen treatment. IVM at 5% oxygen resulted in dramatic changes in the gene expression profile of cumulus cells with expression of more genes being altered in this group. Study of these unique genes may hold the key to discovering the mechanism by which this oxygen concentration mediates such a unique effect.

Likewise, in Chapter 3 it was seen that IVM in a 2% oxygen environment also resulted in the unique effect of increased cell number in resulting blastocysts. The gene expression of cumulus cells following IVM at 2% oxygen was also observed to be altered – but the profile is also different again to that of the 5% oxygen treatment. The study of this cohort of genes (those that are uniquely different between cumulus cells following IVM at 2 and 5% oxygen) may elucidate why such dramatically different outcomes, affecting different stages of development, result from these two treatments. These analyses were not carried out for this thesis due to the constraints of time.

The 2% oxygen incubated cumulus cells are most likely mounting a response to low oxygen mediated by the HIF transcription factors, as evidenced by their gene expression profile (Chapter 5). Whether this ‘hypoxic stress’ response in the cumulus cells could mediate the increase in TE lineage in resulting blastocysts is unknown. It was observed that as oxygen levels during IVM increased, the HIF mediated response is not as apparent in the cumulus cell gene expression profile. Similarly, the increase in cell number in the resultant blastocysts was also less evident.

The oocyte and cumulus cells communicate with one another by the use of paracrine signalling. If oxygen environment during COC maturation was able to alter this communication through impacting on cumulus cell function or the ability of the oocyte to signal (through oocyte secreted factors), this represents a potential mechanism by which subsequent development could be altered. Although this study was able to show alterations in the gene expression of cumulus cells, no direct measure of cumulus cell function or oocyte signalling was taken due to time constraints. However, the examination of these parameters would be carried out as future work.

## **6.4 Future research**

For microarray experiments to be considered significantly relevant, one of two things must occur. Either replicate arrays ( $\geq 3$ ) must be performed with separate RNA pools or the expression profiles of genes of interest must be confirmed by quantitative real time RT-PCR. Future research would include verifying the results reported in Chapter 5 by performing one (or both) of these procedures.

As mentioned previously, long-term studies of the impact of IVM technology on outcomes for the offspring are few. In humans this can be attributed to the fact that IVM has not been used to any great extent clinically, although the numbers of pregnancies and births are on the increase. The children reportedly born from clinical IVM cycles number in the few hundred, and most of these children are less than 5 years of age. Hence, limited information about the longer-term outcomes of IVM is available. Very few experimental animal studies have addressed the impact of IVM on long-term outcomes, and of those performed, studies measuring the effects of oxygen concentration during IVM are non-existent. It is now widely acknowledged, that the environment during early development can influence adult health and disease. It is therefore vital that future work include longer-term studies on the effects of IVM systems on the developmental continuum including fetal, neonatal and adult development and health.

A limitation of this study, as in many others, is that the experiments were carried out in one IVM system. Changing any component in this system could alter the type or extent of the effects seen here. Future studies could investigate the potential for other IVM systems to generate different outcomes under these same oxygen treatments. It would also be wise to keep this in mind when comparing other experimental studies to this one. The use of different medium or media systems during IVM or IVC could alter effects in this way. The availability of glucose to the COC (either through altering concentration in the maturation medium or volume of the medium used for incubation per COC) during IVM has the potential to dramatically alter the outcomes via its involvement in metabolic pathways. Likewise, the system and method used following IVM for zygote culture holds equal importance. The potential for differing outcomes to result from these pre-implantation environments is well documented. Further to this, it would be interesting to investigate the interaction of IVC oxygen concentration with that used for IVM. In this study, 5% oxygen was used for IVF and IVC as a reduced oxygen environment during *in vitro* pre-implantation mouse (and several other species) embryo development has previously been shown to offer improved outcomes (Thompson *et al.* 1990; Umaoka *et al.* 1992; Li and Foote 1993; Gardner and Lane 1996; Dumoulin *et al.* 1999). However, what happens when a COC matured under low oxygen (i.e. 2% oxygen) is fertilised and grown to blastocyst at high oxygen (and vice versa)? It has been shown that a low oxygen environment (2%) during post-compaction embryo culture has effects on the gene expression of mouse embryos particularly in genes known to be HIF responsive (Kind *et al.* 2005). It would be of interest to see if these adverse outcomes were affected by a low oxygen environment during maturation. What would happen if a low oxygen environment was used for maturation and IVC through to blastocyst and the resulting embryos were transferred into a hypoxic recipient? Would adverse outcomes be intensified or would adaptive responses compensate further? Does a constant oxygen environment regardless of concentration throughout IVM, IVC and post-implantation result in improved outcomes for the conceptus? Or, would a system which alters from one extreme oxygen concentration to another induce more dramatic adaptive responses than seen here?

## 6.5 Conclusions

It is likely that a lower than atmospheric oxygen level exists in the follicle where the oocyte undergoes maturation. Oxygen levels in the oviduct and uterus are indeed considerably lower than atmospheric and when this is mimicked during embryo development *in vitro* has been shown to improve subsequent outcomes across many species (e.g. Pabon *et al.* 1989; Thompson *et al.* 1990). The rationale for performing this study was to determine if this principle could also be applied to IVM systems. However, instead of a linear relationship between oxygen concentrations during IVM and subsequent developmental outcomes, a complex array of different effects were seen across development. This study has alluded to the complicated mechanisms by which the oocyte, in combination with its cumulus oophorus, is able to respond and adapt to the oxygen environment in such a way that gross development of both embryo and conceptus are conserved. While this study also goes against historical dogma that low oxygen concentrations are definitively detrimental to oocyte development, it does not refute claims that oxygen availability to the oocyte can have consequences. In fact this study has shown that oxygen availability to the COC has the potential to have effects throughout development including at stages post-implantation. Changes in blastocyst cell number, oocyte metabolism, cumulus cell gene expression and weight of the conceptus were all observed as a direct result of oxygen availability to the oocyte in a finite window during final maturation. The long-term consequences of these effects warrant future investigation and must be acknowledged and considered when using IVM systems such as these either as an experimental model or as a clinical technology.



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## 8 Appendices

## 8.1 Media and solutions

All chemicals were obtained from Sigma unless otherwise stated. All solutions were stored at 4 C for the time recommended. Filtration was via 0.2 µm filter.

### 8.1.1 Stock solutions for IVM/IVF/IVC of mouse oocytes

#### **2x αMEM (makes Bicarbonate buffered and HEPES) stock**

Makes 250 ml of each at 2x concentration.

Dissolve 1 sachet αMEM (Gibco) powder in 200 ml Milli Q (MQ) water. Divide equally between two 250 ml volumetric flasks.

#### For bicarbonate buffered:

To one flask add 1100 mg NaHCO<sub>3</sub>, 25 mg Streptomycin sulphate and 37.5 mg Penicillin G dissolved into a small amount of water (approx 20 ml).

#### For HEPES:

To the other flask add 252 mg NaHCO<sub>3</sub>, 2380 mg HEPES, 25 mg Streptomycin sulphate and 37.5 mg Penicillin G dissolved into a small amount of water (approx 20 ml).

Make both flasks up to 250 ml with MQ water. Measure and adjust pH to 7.2). Filter and store for two weeks.

#### **Stock B**

NaHCO<sub>3</sub>                      2.101 g

Phenol Red                    10 mg

MQ H<sub>2</sub>O                        100 ml

Dissolve in 80 mL MilliQ. Make up to 100 ml in sterile volumetric flask. Filter and store for 3 weeks.

#### **Stock C**

Pyruvic acid                    55 mg

MQ water                        10 ml

Filter and store for 1 week.

**Stock D**

CaCl<sub>2</sub>.H<sub>2</sub>O            1.176 g

MQ Water                40 ml

Filter and store for 3 months.

**Stock G**

D(+) Glucose            1.080 g

MQ Water                40 ml

Filter and store for 3 months.

**Stock L**

Na Lactate 60% syrup    2.832 ml

MQ Water                37.168 ml

Use an unfiltered sterile 1 ml pipette tip with the end cut off; slowly draw the viscous solution up into the tip. Slowly expel the solution into 37.168 ml MQ water. Rinse tip well.

**Stock M**

MgSO<sub>4</sub>.7H<sub>2</sub>O            1.972 g

MQ Water                40 ml

Sterilise and store for 3 months.

**Stock 10x KSOM**

Component	Weight	Final concentration
NaCl	5.552 g	950 mM
KCl	0.178 g	25 mM
KH <sub>2</sub> PO <sub>4</sub>	0.006.8 g	0.5 mM
EDTA	0.003.8 g	0.1 mM
MQ H <sub>2</sub> O	100 ml	

Dissolve chemicals in 100 ml MQ H<sub>2</sub>O and mix well. Sterilise and store for 3 months.

### **8.1.2 Media for IVM/IVF/IVC of mouse oocytes**

#### **1x $\alpha$ MEM (bicarbonate buffered and HEPES)**

For bicarbonate buffered 1x:

Makes 25 ml.

To 11.88 ml of MQ water, add 11.88 ml of  $\alpha$ MEM bicarbonate buffered 2x stock and 1.25 ml of non-heat treated FCS (NHT FCS). Filter and store for 1 week.

For HEPES 1x:

Makes 50 ml.

To 23.75 ml of MQ water, add 23.75 ml  $\alpha$ MEM HEPES 2x stock and 2.5 ml of NHT FCS. Filter and store for 1 week.

#### **Culture $\alpha$ MEM (50 mIU FSH)**

Makes 1 ml.

To 995  $\mu$ l of  $\alpha$ MEM bicarbonate buffered 1x add 5  $\mu$ l (50mIU) of rhFSH (Puregon; Organon, Sydney, Australia).

#### **$\alpha$ MEM for IVF (3 mg/ml BSA)**

Makes 5 ml.

To 2.5 ml of MQ water add 2.5 ml  $\alpha$ MEM bicarbonate buffered 2x stock and 15 mg BSA. Do not shake. Filter once BSA is dissolved.

#### **KSOM for embryo culture**

Makes 10 ml.

To 7.602 ml of MQ water add the following-

1 ml 10x KSOM stock

1 ml stock B

200  $\mu$ l stock L



84 µl stock D

50 µl Glutamax

40 µl stock C

14 µl stock G

10 µl stock M

Osmolarity should be between 250 and 260 mOsm and pH around 8. Add 10 mg BSA and allow to dissolve (without shaking). Filter and store for 1 week.

#### Chemical composition of KSOM-

<u>Chemical</u>	<u>Final concentration</u>
NaCl	95 mM
KCl	2.5 mM
KH <sub>2</sub> PO <sub>4</sub>	0.05 mM
EDTA	0.01 mM
NaHCO <sub>3</sub>	25 mM
Pyruvate	0.2 mM
Glucose	0.2 mM
Na Lactate	10 mM
CaCl <sub>2</sub> .2H <sub>2</sub> O	1.7 mM
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.2 mM
Glutamax I	1 mM
BSA-FAF (ICP)	1 mg/ml

#### Stock composition-

See stock recipes for more detail.

<u>Stock</u>	<u>Final concentration</u>	<u>Chemical</u>
Stock H	250 mM	Hepes
Stock B	250 mM	NaHCO <sub>3</sub>
Stock C	50 mM	Pyruvate
Stock L	500 mM	Na Lactate
Stock D	200 mM	CaCl <sub>2</sub> .2H <sub>2</sub> O
Stock M	200 mM	MgSO <sub>4</sub> .7H <sub>2</sub> O
Stock G	150 mM	Glucose
Glutamax I	200 mM	L-Alanyl-L-Glutamine

### **8.1.3 Solutions for orcein staining**

#### ***Oocyte fix***

1 part 100% ethanol

3 parts acetic acid

#### ***Orcein stain***

1 g orcein

45 ml acetic acid

Dissolve with gentle heat. Add 55 ml of water. Filter and store in a glass bottle.

#### ***Clearing solution***

1 part glycerol

1 part acetic acid

3 parts MQ water

### **8.1.4 Solutions for immunohistochemistry**

#### ***PBS 10x stock***

Makes 100 ml.

8.0 g NaCl

0.20 g KCl

1.43 g Na<sub>2</sub>HPO<sub>4</sub>

0.24 g KH<sub>2</sub>PO<sub>4</sub>

Dissolve in < 100 ml of MQ water, and then adjust to 100 ml and pH to 7.2.

Use at 1x.

#### ***PBS/Gly/BSA***

Makes 100 ml.

1.125 g Glycine

0.3 g BSA (low grade)

Dissolve in < 100 ml PBS 1x, and then make up to 100 ml with PBS.

***PBS Triton X-100 (0.1%)***

Makes 100 ml.

0.1 ml Triton X-100

100 ml PBS 1x

Leave to dissolve. Gentle heating or shaking may be required.

**8.1.5 Solutions for TUNEL**

***PBS 10x stock***

Makes 100 ml.

7.597 g NaCl

1.246 g Na<sub>2</sub>HPO<sub>4</sub>

0.480 g Na<sub>2</sub>H<sub>2</sub>PO<sub>4</sub>

Dissolve in < 100 ml MQ water, and then make up to 100 ml and pH to 7.4.

Use at 1x.

***3% PVP stock***

Makes 100 ml.

3 g PVP in < 100 ml MQ water made up to 100 ml.

***PBS/PVP***

Makes 100 ml.

10 ml 3% PVP stock

90 ml PBS 1x

## 8.2 Full version of papers arising from this work

### ***Oxygen concentration during mouse oocyte in vitro maturation affects embryo and fetal development***

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**BACKGROUND:** Little is known of how the oxygen environment in the ovarian follicle affects oocyte and embryo development, but this has an important impact on the conditions used for *in vitro* maturation (IVM) of oocytes. We investigated the effect of varying oxygen concentrations during IVM on subsequent pre and post-implantation development.

**METHODS:** IVM of mouse cumulus-oocyte complexes (COCs) was performed under 2, 5, 10 or 20% O<sub>2</sub> (6% CO<sub>2</sub>, balance N<sub>2</sub>). *In vivo*-matured COCs were collected post ovulation. Embryos were generated by IVF and culture. Blastocyst development, cell number and apoptosis were assessed, and fetal and placental outcomes analysed following embryo transfer at day 18 of pregnancy.

**RESULTS:** Oxygen concentration during IVM did not affect oocyte maturation or subsequent fertilization, cleavage and blastocyst development rates. Maturation of oocytes under 2% O<sub>2</sub> increased blastocyst trophoctoderm cell number compared with all groups and numbers at 5% were higher than 20% (both  $P < 0.05$ ). Percentage of apoptotic cells was increased in blastocysts developed from 2% O<sub>2</sub>-matured oocytes, compared with maturation at 5% O<sub>2</sub> or *in vivo* ( $P < 0.05$ ). Rates of embryo implantation and development into a viable fetus were not altered by IVM oxygen. However, fetal weight was reduced following oocyte maturation at 5% O<sub>2</sub> compared with 20% O<sub>2</sub> and maturation at 5% O<sub>2</sub> also reduced placental weight, when compared with *in vivo*-matured oocytes (both  $P < 0.05$ ).

**CONCLUSIONS:** Level of O<sub>2</sub> exposure during oocyte maturation can alter the cellular composition of blastocysts, but these changes in cell number do not correlate with the altered fetal and placental outcomes after transfer.

*Key words:* *in vitro* maturation/oocyte maturation/oxygen/trophoctoderm

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## **In Vitro Maturation of Mammalian Oocytes: Outcomes and Consequences**

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### **ABSTRACT**

The application of in vitro maturation (IVM) of oocytes as a technology to assist animal production and clinical infertility treatment remains poor because of the reduced developmental competence of oocytes after IVM, despite several decades of research. Reduced meiotic maturation rates, fertilization rates, and blastocyst production reveal short-term developmental insufficiency of oocytes when compared with in vivo-matured counterparts. However, there is an increasing body of evidence that demonstrates the capacity of IVM efficiency to be improved, some of which is reviewed here. Of more concern is the role that IVM of oocytes may play in causing or accentuating long-term development and health of fetuses and neonates after in vitro production of embryos and embryo transfer. This is a largely unexplored area, yet the application of such techniques, especially the safety of clinical IVM, is significant and requires monitoring before acceptance as a routine procedure.

### **KEYWORDS**

Maturation - oocyte - outcomes

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