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Metabolism throughout follicle and oocyte development in mammals

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ABSTRACT Metabolic studies of mammalian embryos started with the development of *in vitro* culture systems more than 40 years ago. More recently, metabolic studies have begun to shed light on the requirements of growing oocytes/follicles from the earliest stages of folliculogenesis. While growing oocytes preferentially metabolise pyruvate over glucose, the somatic compartment of ovarian follicles is more glycolytic. The metabolic preferences of the oocyte are reflected in the early zygote, which becomes increasingly dependent on glycolytic energy production as development progresses to the blastocyst stage. Furthermore, the intricate metabolic relationship between each oocyte and its somatic surroundings is critical for oocyte growth and developmental competence. Measurements of amino acid turnover in bovine oocytes indicate that glutamine, arginine and leucine are consistently depleted, while alanine is produced, showing similarities with amino acid turnover in preimplantation embryos. Amino acid profiling is a good predictor of embryo quality and might also turn out to be a predictor of oocyte developmental competence. Finally, recent studies have uncovered lipid metabolism in oocytes and early embryos, suggesting that endogenous fatty acids might be used for energy production. Together, metabolic studies have revealed the multiplicity of energetic substrates used by oocytes and early embryos, and suggest that the versatility of the metabolic pathways available for energy production is key for high developmental potential. Metabolic studies of early embryos are now being applied to follicle culture, and the goal of describing the metabolome of the growing oocyte in its follicle is now very attainable.

KEY WORDS: *oocyte, follicle, metabolism, carbohydrate, amino acid, lipid*

Introduction

The revolution of metabolomics is promising to deliver an exhaustive description of the oocyte's metabolome, and to reveal the metabolome of a healthy oocyte in order to discriminate (non invasively) which gamete has the greatest chance of producing a successful pregnancy. Numerous studies have described the metabolic pathways operating in oocytes and follicles. Metabolomic studies must also describe the metabolite fluxes in the oocyte in order to decipher the respective contribution of each metabolic pathway during oocyte development. The abundance of biochemical studies published since the development of *in vitro* culture of mammalian embryos in the 1960s, provides the opportunity to tentatively start describing the oocyte metabolome to possibly direct future studies employing modern techniques. Metabolic studies -which initially used radiolabelled substrates- made it possible to define the metabolites that mammalian oocytes and embryos require. More recently, amino acid analysis of spent culture media has revealed the usage of different amino acids by oocytes (Hemmings *et al.*, 2012) and embryos (Sturmey *et al.*, 2008) in a range of species,

Abbreviations used in this paper: AMP, adenosine monophosphate; ATP, adenosine triphosphate; CC, cumulus cell; COC, cumulus-oocyte complex; CPT1, carnitine palmitoyl transferase 1; Cx, connexin; EGA, embryo genome activation; EGF, epidermal growth factor; G6PDH, glucose-6-phosphate dehydrogenase; GC, granulosa cells; GSH, glutathione; GV, germinal vesicle; GVBD, germinal vesicle breakdown; HBP, hexosamine byosinthetic pathway; hCG, human chorionic gonadotropin; HPLC, high performance liquid chromatography; IVM, *in vitro* maturation; LDH, lactate dehydrogenase; LH, lutheinising hormone; MII, metaphase II; NADH, nicotinamide adenine dinucleotide (reduced form); NADPH, nicotine adenine dinucleotide phosphate (reduced form); Oxphos, oxidative phosphorylation; PGC, primordial germ cell; PPP, pentose phosphate pathway; TCA cycle, tricarboxilic acid cycle.

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and is providing insights into the amino acids utilized by healthy oocytes (Hemmings *et al.*, 2012). Indeed, in pre-implantation embryos the quantification of amino acid and energy metabolism is emerging as a potential non-invasive tool with which to assess embryo developmental competence and even pregnancy outcome (Sturmey *et al.*, 2008; Picton *et al.*, 2010; Gardner *et al.*, 2011).

Mammalian follicle and oocyte metabolism has been studied mainly in antral follicles and fully grown oocytes. Little is known about follicle/oocyte metabolism at earlier stages of folliculogenesis. Studies in murine follicles and oocytes, suggest that their metabolic profiles change dynamically as follicle development progresses (Harris *et al.*, 2007; Harris *et al.*, 2009). A better understanding of the metabolism of early follicles and oocytes is important as they represent the greatest proportion of follicles in the female ovaries. Its study is of value for the development of better culture systems for *in vitro* growth and *in vitro* production of oocytes, techniques applied in fertility preservation and stem cell production. In this review we aim to provide an overview of the oocyte and follicle metabolism throughout oogenesis. Metabolism of early embryos will also be mentioned, but we refer the interested reader to reviews on embryo metabolism (Johnson *et al.*, 2003; Dumollard *et al.*, 2009; Leese, 2012). After briefly describing oogenesis and folliculogenesis, we review studies addressing carbohydrate and amino acid metabolism in follicles and oocytes to emphasise how the metabolic cooperativity between the oocyte and supporting cells underlie such a complex and changing metabolism. Finally, the possibility that endogenous fatty acids might support oocyte development will be discussed.

Oogenesis and folliculogenesis: an overview

Oocyte development begins in the female mammalian foetus with the differentiation of primordial germ cells (PGC). Proliferating PGC migrate towards the nascent genital ridges, where they differentiate into oogonia, before entering meiosis to become primary oocytes (McLaughlin and McIver, 2009). During post natal life, oocytes undergo growth and maturation, stockpiling mRNAs, proteins, metabolic substrates and organelles. Such maternal stores must support fertilization, unpacking of paternal genome, karyogamy and early cleavage division of the zygote until embryo genome activation (EGA) begins. Primary oocytes, which remain arrested at diplotene of the 1st meiotic prophase, become enveloped by a single layer of flattened pre-granulosa cells and a basement membrane to form primordial follicles (Fig. 1). The ovarian population of primordial follicles are continuously depleted by atresia or stimulated to grow during follicle activation (McLaughlin and McIver, 2009). Following growth activation, primordial follicles become primary follicles, which are characterised by a full layer of proliferative cuboidal granulosa cells (GC) (Picton, 2001) (Fig. 1). Over the lengthy process of follicular growth, GC continue to proliferate, the theca layer develops which provides the follicle with an independent blood supply (Young and McNeilly, 2010), and follicles pass through the secondary, preantral and antral stages before final maturation and ovulation (Binelli and Murphy, 2010) (Fig. 1). The antral cavity forms when follicles reach a diameter between 200-500μm depending on the species (Picton *et al.*, 1998). It becomes filled with follicular fluid, which acts as a source of oxygen, buffering molecules, carbohydrates, amino acids, growth factors, hormones and other molecules (Sutton *et al.*, 2003b). In addition, the formation of the antrum leads to the differentiation of GC into two spatially and functionally distinct populations: the mural granulosa cells, which line the basement membrane and are characterised by their endocrine role; and the cumulus cells, which are intimately associated with the oocyte and support its metabolism and maturation. Both cell types form the cumulus-oocyte complex (COC) (Fig. 1).

Within the growing follicle, the oocyte undergoes a 100-300-fold increase in volume (Griffin *et al.*, 2006), which is largely completed around the time the antrum is formed, and is supported by an increasing number of surrounding GC (Griffin *et al.*, 2006) (Fig. 1). The ooplasm of the growing oocyte accumulates glycogen granules, lipid droplets -especially in farm animals (Sturmey *et al.*, 2009)-, proteins and mRNA. The endoplasmic reticulum and Golgi complexes are abundant and undergo structural changes and redistribution, reflecting the high synthetic activity of the oocyte (Fair *et al.*, 1997; Picton *et al.*, 1998). The number of vesicles and ribosomes increases, cortical granules appear and glycoproteins are exported to form the zona pellucida (Fair *et al.*, 1997; Picton *et al.*, 1998). Glutathione (GSH), a glutamate-cysteine-glycine tripeptide that protects the oocyte from oxidative stress and is re-

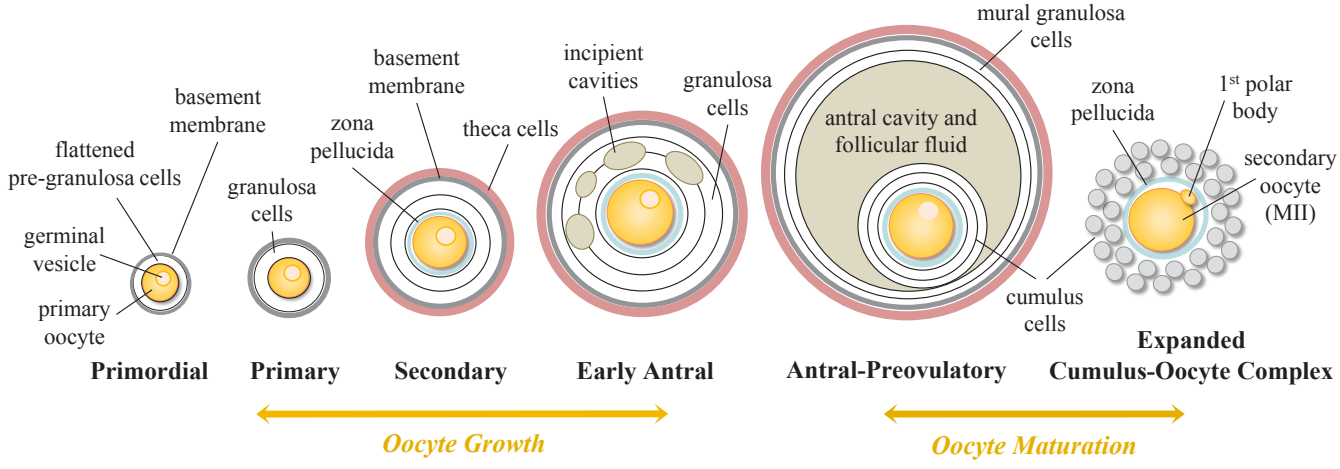

Fig. 1. Simplified representation of mammalian folliculogenesis and oocyte growth. *Follicle developmental stages are indicated, and the different cell layers at each stage are represented as full lines without cellular divisions.*

quired for the decondensation of the sperm nucleus and formation of the male pronucleus, also accumulates during oocyte growth and maturation (reviewed by Luberda (2005)). Mitochondria also replicate during oogenesis, and their number rises from about 200 per oogonia to 6,000 per oocyte in primordial follicles, and reaches up to 300,000 – 400,000 in the mature human oocyte (Jansen and de Boer, 1998). It is hypothesised that such a rise in mitochondria number compensates for their apparent lack of maturity and activity. In turn, the low activity of individual mitochondria is thought to minimise oxidative stress (Dumollard *et al*., 2006). Mitochondrial replication then ceases, and resumes only after implantation (reviewed by Jansen and de Boer (1998) and Dumollard *et al*.(2006)). The expansion of the mitochondrial population during oogenesis is essential for supplying all the mitochondria of the preimplantation embryo, and as such it is vital for preimplantation development.

Selection and final growth of the follicle/s destined to ovulate is mediated by the preovulatory surge of gonadotropins, which triggers COC expansion, gap junction loss, and ultimately oocyte maturation and ovulation (Binelli and Murphy, 2010). Oocyte maturation is comprised of both, nuclear and cytoplasmic maturation, and both must be completed for the gamete to acquire developmental competence (Eppig, 1996). Nuclear maturation consists of the nuclear changes associated with the resumption of meiosis I (germinal vesicle breakdown (GVBD), progression to metaphase II (MII) and extrusion of the first polar body, and arrest at MII (for a review see (Tripathi *et al.,* (2010)). In contrast, cytoplasmic maturation comprises the acquisition of mechanisms for sperm penetration and polyspermy block, calcium release and exocytosis of cortical granules, decondensation of the sperm chromatin, pronuclei formation, and support of the first embryonic divisions (Ajduk *et al*., 2008; Ferreira *et al*., 2009). Post-transcriptional modification of stable mRNAs stored during oocyte growth, protein synthesis and post-translational regulation are also a critical part of cytoplasmic maturation (Picton *et al*., 1998). In addition, oocyte mitochondria redistribute during GVBD and progression to MII, and altered mitochondrial redistribution is associated with lower oocyte developmental potential. The multiple and pervasive functions of mitochondria in oocytes and early embryos have been reviewed recently and will not be discussed further (Dumollard *et al*., 2006; Dumollard *et al*., 2007; Dumollard *et al*., 2009; Van Blerkom, 2011).

Oocyte developmental competence has been shown to be acquired progressively during oocyte growth in different mammalian species (for a review see (Trounson *et al*., 2001). Many of the dynamic processes occurring during oocyte growth and maturation require energy, and oocytes and follicles can exploit a variety of metabolites to support their energetic and anabolic needs.

Carbohydrate metabolism throughout folliculogenesis

Energy metabolism of PGC and oogonia was suggested to be mainly anaerobic and glycolytic, thus avoiding oxidative damage (Jansen and de Boer, 1998). PGCs contain a large nucleus, few organelles, abundant glycogen and occasional lipid droplets, which could provide a source of energy during their migration (Motta *et al*., 1997). However, PGCs have also been shown to preferentially oxidise pyruvate over glucose (Brinster and Harstad, 1977) indicating aerobic oxidation in PGCs. Besides, primordial follicles readily produce lactate, and consume 2-fold more pyruvate than glucose, indicating that both glycolysis and mitochondrial pyruvate oxidation operate at this stage, and that pyruvate might be the main energy substrate (Harris *et al*., 2009). Oocytes at the earliest stages of development are not quiescent. Indeed, when the substrate preference by oocytes of a diameter approximately corresponding to those within primordial follicles was studied, no oxidation of glucose to CO_2 was detected, only pyruvate oxidation was observed (Eppig, 1976); and at a rate similar to that of primordial follicles as described by Harris *et al.,* (2009). Thus, it appears that the small number of pre GC in primordial follicles could be responsible for the little glucose consumption and lactate production by the intact follicle, while pyruvate is consumed by denuded primordial oocytes (Harris *et al*., 2009). Species-specific differences in substrate preferences might nevertheless exist, as shown for fully grown oocytes (Krisher *et al*., 2007).

With the initiation of follicle and oocyte growth, a highly active period starts. Unlike resting primordial follicles, which are located in the poorly vascularised outer ovarian cortex, growing follicles are found in the well vascularised cortico-medullary border (Picton, 2001). In addition, the energetic demands of the growing oocyte seem to be reflected in the increasing pyruvate and oxygen consumption by murine oocytes throughout the growth period (Harris *et al*., 2009). Indeed, when data was corrected for oocyte volume, oocytes from primary follicles showed a particularly high metabolic turnover (Harris *et al*., 2009) (Fig. 2 B,C). Glucose uptake was not detected at any stage of murine oocyte growth (Harris *et al*., 2009), indicating a low glycolytic activity. This is in agreement with studies in fully grown oocytes, where although glucose consumption and metabolism by denuded oocytes was detected, glucose represented a minor energetic substrate (Rieger and Loskutoff, 1994; Sutton-McDowall *et al*., 2010). Follicle glucose consumption and lactate production rates increase as murine follicles develop *in vitro* from primary/secondary stages to antral and ovulatory-like stages (Boland *et al*., 1994a; Harris *et al*., 2007) (Fig. 2A). Nevertheless, the low ratio of glucose consumption to lactate production suggests that glycolysis contributes poorly to energy production during early preantral stages (Harris *et al*., 2007). This pattern appears to change around the time of antrum formation, with antral follicles being regarded as predominantly glycolytic. Such increased glycolytic activity might be related to low oxygen availability, initiation of oestrogen synthesis and increasing energy demands (Boland *et al*., 1993; Boland *et al*., 1994b; Harris *et al*., 2007).

When the growing follicle reaches a size at which supply from theca capillaries could be compromised, because of the thickening and nutritional requirements of the GC layer, the follicular fluid acts as a source of oxygen and nutrients (Redding *et al*., 2007). Mathematical modeling was used to study the mean oxygen concentration in human follicles; and predicted a low oxygen concentration within large preantral follicles, yet sufficient to reach the oocyte (Redding *et al*., 2007). Other studies in the human (Redding *et al*., 2008) and bovine (Clark and Stokes, 2011) predict a sharp rise in intrafollicular oxygen with antrum formation, peaking at the end of the early antral stage before decreasing through the antral to preovulatory stages, and to finally increase around the time of ovulation. Predicted and actual measurements of oxygen concentration in antral follicles are similar to those in venous blood (Redding *et al*., 2008). Thus, the oxygen supply to the oocyte may only be limited at the late preantral to early antral stage and at the early preovulatory stage (Redding *et al*., 2007, 2008). Follicle angiogenesis at these stages could be crucial for oocyte oxygen-

ation and development (Van Blerkom, 2000).

The final stages of folliculogenesis *in vivo* are marked by the preovulatory surge of gonadotropins. *In vitro*, gonadotropins cause a dramatic increase in follicle glucose uptake and lactate production (Roberts *et al.*, 2004; Sutton-McDowall *et al.*, 2004; Harris *et al.*, 2007) (Fig. 2A). Such actions are likely mediated by up-regulation of glucose transporters (Kol *et al.*, 1997; Roberts *et al.*, 2004) and glycolytic enzymes (Downs *et al.*, 1996; Roy and Terada, 1999). Glucose metabolism is also influenced by insulin and ovarian growth factors (Roy and Terada, 1999). Yet, the proportion of glucose metabolised by glycolysis has been shown to diminish following follicle/COC maturation, suggesting that some glucose is metabolised through an alternative pathway/s (Sutton *et al.*, 2003a; Sutton-McDowall *et al.*, 2004; Harris *et al.*, 2007). One of these pathways is probably the PPP (Downs *et al.,* 1998). This is supported by the relative high activity of the PPP rate limiting enzyme glucose-6-phosphate dehydrogenase (G6PDH) compared to the glycolytic enzyme phosphofructokinase in bovine oocytes during in vitro maturation (IVM) (Cetica *et al.,* 2002). Yet, a decrease in G6PDH activity in fully grown oocytes has been reported, and suggested to be linked to oocyte cytoplasmic maturation (Su *et al.,* 2012; Ishizaki *et al.,* 2009). Furthermore, it is well established that glucose is required to support FSH-dependent cumulus expansion and oocyte maturation (Fagbohun and Downs, 1992), while denuded mouse oocytes require pyruvate or oxaloacetate to sustain spontaneous nuclear maturation (Biggers *et al.*, 1967; Downs and Hudson, 2000). The oocyte energetic requirements for resumption of meiosis appear to be met by an increase in oxidative metabolism around the time of GVBD; pyruvate being used as the main substrate, and supplemented by glutamine and glycine which can feed into the TCA cycle (Zuelke and Brackett, 1993; Rieger and Loskutoff, 1994; Downs *et al.,* 2002; Harris *et al.,* 2007). Metabolites such as glucose and fatty acids are also thought to play a key role in bovine and porcine oocyte maturation (Krisher *et al.*, 2007; Sturmey *et al.*, 2009).

Within the follicle glucose can be metabolised through: (i) glycolysis -producing adenosine triphosphate (ATP) and pyruvate or lactate; (ii) the pentose phosphate pathway (PPP) -providing precursors of purine nucleotides and nicotine adenine dinucleotide phosphate hydrogen (NADPH) for biosynthetic pathways and antioxidant defence. Although metabolic flux though the PPP might be low, it is thought to be key for COC maturation; (iii) the hexosamine biosynthetic pathway (HBP) -by which glucose and glutamine are involved in protein glycosylation as well as hyaluronic acid synthesis for cumulus expansion; and (iv) the polyol pathway producing sorbitol and fructose -whose role remains largely unknown (for a review see (Sutton-McDowall *et al.*, 2010)). Glucose-derived or extracellular pyruvate can be metabolised in the mitochondria through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (oxphos), which produces most of the ATP in the oocyte (Dumollard *et al.*, 2007; Dumollard *et al.*, 2009). Lactate, highly abundant in follicular fluid and the reproductive tract (Harris *et al.*, 2005), is oxidised to pyruvate by cytosolic lactate dehydrogenase (LDH). Interestingly, such lactate-derived pyruvate does not fuel mitochondrial ATP production in denuded mouse oocytes, instead lactate is important for regulation of the cytosolic redox state (Dumollard *et al.*, 2007; Dumollard *et al.*, 2009). Mitochondria also metabolise ketone bodies originating from deamination of amino acids such as leucine, and are responsible for the catabolism of fatty acids through β-oxidation. While GC are mainly

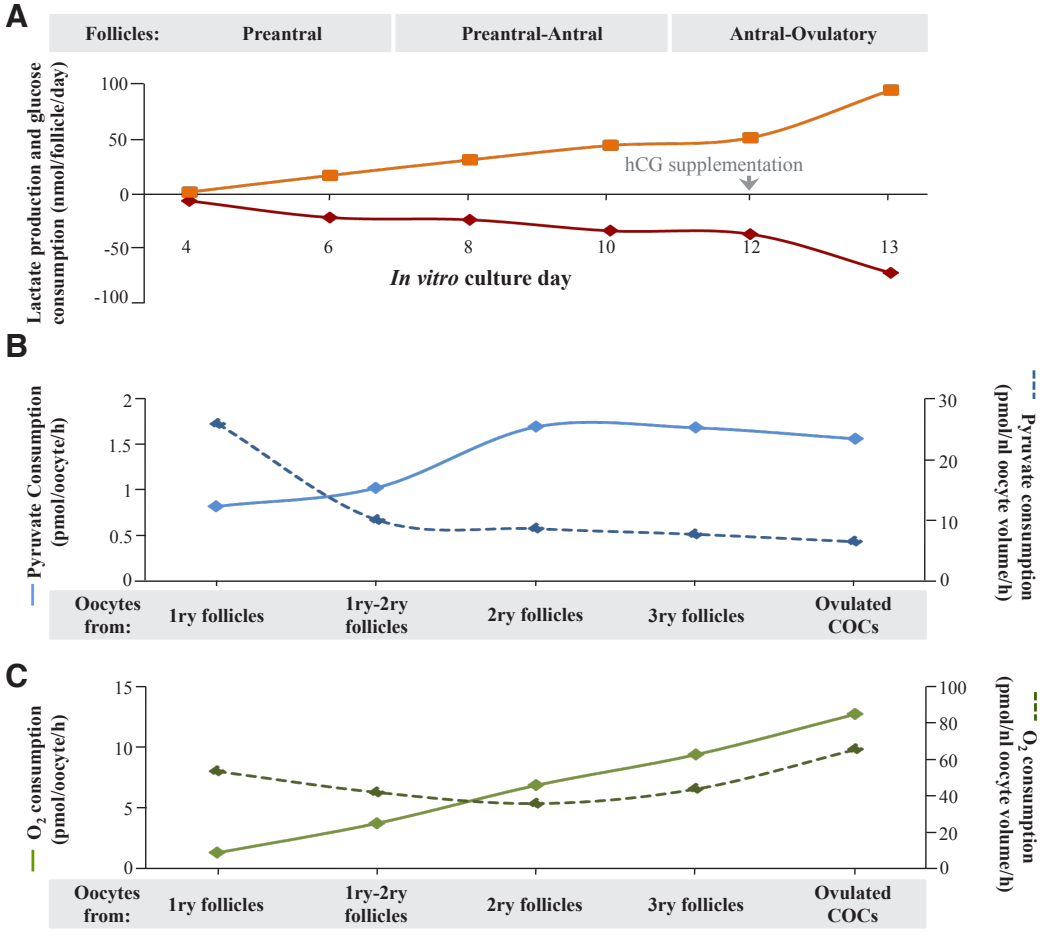

Fig. 2. Carbohydrate metabolism of murine follicles and oocytes throughout development. (A) *Lactate production (positive values) and glucose consumption (negative values) by* in vitro *grown follicles, which were at the late primary to secondary stage at the beginning of culture. Follicle developmental stages and culture days are indicated (adapted from Harris* et al.*, 2007).* **(B)** *Pyruvate consumption and* **(C)** *oxygen consumption by oocytes from follicles at different stages. Follicle stages are indicated. Dashed lines indicate data corrected for oocyte volume (adapted from Harris* et al.*, 2009).*

glycolytic, oocyte energy metabolism relies mainly on mitochondrial oxidation of extracellular pyruvate as well as metabolites supplied by the somatic compartment. Mitochondrial oxidative metabolism is the major oxygen consumer in the follicle and the oocyte, and -besides mitochondrial oxphos- oxygen is required for synthesis of cholesterol and steroidogenesis in GC of antral follicles (Boland *et al*., 1994a), and for β-oxidation of intracellular lipid stores (see below). The requirement for oxygen is illustrated by the detrimental effect anoxic culture conditions have upon murine follicle development and steroidogenesis (Boland *et al*., 1994a). Disruption of the TCA cycle in murine oocytes resulted in compromised nuclear maturation and developmental competence (Johnson *et al*., 2007). There is now strong evidence that mitochondrial function supports ATP production and Ca^{2+} homeostasis, and sets the cellular redox state in the mouse oocyte and embryo; and such functions are key to the oocyte and embryo developmental competence (for a review see (Johnson *et al*., 2003; Dumollard *et al*., 2007; Dumollard *et al*., 2009; Van Blerkom, 2011).

Cumulus expansion represents the end of the oocyte-CC metabolic cooperativity. The oocyte and preimplantation embryo then rely on internal reserves and the external medium for provision of metabolites. It is important to bear in mind the changing environment from that within the follicular fluid, through that in the post ovulatory oviduct fluid and later in the uterus (Fischer and Bavister, 1993; Harris *et al*., 2005). There is no specific blood supply following ovulation and until well after implantation (Burton *et al*., 2010), and significant variations in oxygen, carbohydrate and amino acid concentration along the reproductive tract have been reported (Fischer and Bavister, 1993; Harris *et al*., 2005; Hugentobler *et al*., 2007).

Changes in (i) the environment characteristics, with both restrictive and facilitative action (e.g. oxygen concentration and nutrient supply); and (ii) the increasing embryo requirements for energy and metabolites to support cell division, EGA and blastocoel formation, are reflected in the major changes in embryo metabolism during the preimplantation period. Namely increasing metabolic activity and gradual shift towards greater importance of glucose metabolism (for a review see (Johnson *et al*., 2003) and (Leese, 2012)). A part from this general pattern, there are important species-specific differences in the use of metabolic substrates and the relative importance of pyruvate and glucose metabolism (Swain *et al*., 2002).

Amino acid metabolism supports oocyte growth and cytoplasmic maturation

Amino acids serve as substrates for the synthesis of proteins, nucleotides (glutamine, aspartate, glycine), GSH (glutamate, cysteine, glycine), glycoproteins, hyaluronic acid (glutamine bound to glucose through the HBP), and signalling molecules such as nitric oxide (arginine). They also play important roles as energy substrates (glutamine, glycine, and others), pH and osmolarity regulators (glycine, alanine, glutamine, and others), heavy metal chelators (glycine), donors of methyl groups (methionine), in the secretion of ammonia (alanine) and in anaplerosis (aspartate, glutamate, leucine, lysine, isoleucine and others) (Dumollard *et al*., 2007; Sturmey *et al*., 2008).

Growing oocytes are characterized by a high RNA and protein synthetic activity (Picton *et al*., 1998). Protein synthesis is also particularly high during the early stages of oocyte maturation (Tomek *et al*., 2002). For example, the RNA content and the absolute rate of protein synthesis increase 300-fold and 38-fold respectively, during the growth phase of murine oocytes (Schultz *et al*., 1979; Sternlicht and Schultz, 1981). Glutamine, aspartate, glycine and ribose-5-phosphate (product of glucose metabolism through the PPP) are required for *de novo* synthesis of purine and pyrimidine nucleotides for mRNA synthesis. A part from protein and mRNA synthesis, some amino acids can also serve other purposes in the oocyte. Indeed, part of the ATP demands could be met by oxidative metabolism of glutamine and glycine through the TCA cycle (Zuelke and Brackett, 1993; Rieger and Loskutoff, 1994).

In comparison to carbohydrate metabolism, and despite their important roles, there is little information about amino acid uptake and metabolism by follicles and oocytes, especially at the earlier stages of folliculogenesis. Studies measuring amino acids in follicular fluid and the reproductive tract provided valuable information about the available substrates, showing significant levels of amino acids. For example glutamine, glycine and alanine are consistently among the amino acids at highest concentrations throughout the reproductive tract in different species (Harris *et al*., 2007; Hugentobler *et al*., 2007). Studies investigating amino acid metabolism have mainly focused on the embryo (Van Winkle, 2001; Sturmey *et al*., 2008), while less information is available from oocytes (Pelland *et al*., 2009; Hemmings *et al*., 2012). In addition, although about 18 amino acid-selective transport systems are thought to operate in mammalian embryos, indicating their capacity for amino acid uptake (Van Winkle, 2001), only a few have been identified during oocyte growth and maturation (Pelland *et al*., 2009). Overall, embryos appear to consume glutamine and arginine and produce alanine consistently during preimplantation development, yet a more specific description of amino acid utilisation depends upon the culture conditions, the developmental stage and the species studied (see for example pig: (Booth *et al*., 2005); bovine: (Partridge and Leese, 1996); human:(Houghton *et al*., 2003)).

In the bovine, LH has been shown to increase glutamine oxidative metabolism by oocytes and COCs (Zuelke and Brackett, 1993). Addition of glutamine to bovine IVM media also appears to promote oocyte nuclear maturation (Bilodeau-Goeseels, 2006),

TABLE 1

AMINO ACID TRANSPORT AND TURNOVER IN GROWING AND FULLY GROWN OOCYTES

Growing Oocytes	Oocytes Undergoing Maturation
Increasing saturable transport of Ala, Lys, Leu, Gln, Arg [(1)]	Peak in saturable transport of Gly, Tau, cystine [(1)]
Uptake of Ala, Lys, Gly and Tau is enhanced by follicle cells (Ala, Lys and Tau uptake by fully grown oocytes also enhanced by CC) [(1,2)]	Consumption of Gln and Arg, and production of Ala and Gly by mature *bovine* oocytes [(3)]
Leu uptake is inhibited by the presence of follicular cells [(1)]	Supplementation of IVM media with some amino acids appears to have beneficial effects upon oocyte maturation and developmental potential in different species (i.e. Gln, Cys) [(4)]

Data refers to murine oocytes unless otherwise stated. Metabolism of murine oocytes throughout development (1: Pelland *et al*., 2009; 2: Eppig *et al*., 2005; 3: Hemmings *et al*., 2012; 4: see text for references)

while supplementation of defined IVM media with non-essential and essential amino acids increases oocyte maternal mRNA levels and enhances embryo development (Watson *et al.*, 2000). In the pig, defined IVM media supplemented with glutamine, aspartate and valine enhanced the rate of monospermic fertilization; while most of the amino acids studied improved male pronucleus formation, arginine and alanine had a beneficial effect on embryo development (Hong and Lee, 2007). In the mouse, the use of glutamine as a sole energy substrate was shown to be sufficient to initiate GVBD, but not to reach MII (Downs and Hudson, 2000), which could be related to an insufficient activity of the PPP (Sutton *et al.*, 2003b).

Three recent studies have provided new information regarding the amino acid turnover of oocytes and follicles over growth and maturation (see Table 1). First, Chand and Legge (2011) measured the uptake of radiolabelled leucine in mouse follicles and reported an increase in the rate of leucine uptake as follicles developed from pre-antral to late antral stages *in vitro*. A reduction in transport was observed as follicles attained the pre-ovulatory stages (Chand and Legge, 2011). Second, Pelland *et al.,* (2009) characterised the transport systems for nine amino acids (aspartate, glutamine, glycine, arginine, alanine, leucine, lysine, cystine, and taurine) through growth and maturation of murine oocytes. Leucine, arginine and lysine were transported at the highest rates (Pelland *et al.*, 2009). Three different patterns were identified: (i) Aspartate did not show saturable transport and was transported at a very low rate, especially after GVBD. (ii) Saturable transport for alanine, lysine, leucine, glutamine and arginine increased during oocyte growth, and reached a plateau or decreased from MI to MII, suggesting a role during the energy demanding and stockpiling process of oocyte growth, cytoplasmic maturation and GVBD. (iii) Saturable transport for glycine, cystine and taurine was very low or absent during growth and peaked in MI and MII oocytes (Pelland *et al.*, 2009). Such patterns of amino acid transport may reflect the demand for glycine and cystine for the major GSH synthesis occurring during oocyte maturation (Luberda, 2005; Pelland *et al.*, 2009). Moreover, six different amino acid transport systems were identified both in growing and mature oocytes. And follicular cells were shown to enhance the uptake of glycine, alanine, lysine and taurine by oocytes (Pelland *et al.*, 2009). Finally, Hemmings *et al.,* (2012) quantified the amino acid profile (i.e. depletion and appearance rates) by bovine MII oocytes after IVM. Glutamine, arginine and asparagine were depleted at the highest rates, while alanine and glycine were released into the media. Similar to studies carried out in embryos, oocytes with higher developmental potential after IVF and those of poorer quality presented different amino acid profiles (Hemmings *et al.*, 2012). Overall, poorer quality was related to higher amino acid turnover, in agreement with Leese's "quiet embryo hypothesis" (Leese *et al.*, 2008). For example, oocytes that failed to cleave depleted more glutamine, released more alanine and presented higher total amino acid depletion, appearance and turnover than oocytes able to cleave (Hemmings *et al.*, 2012). Furthermore, these data was used to predict fertilisation and cleavage potential (Hemmings *et al.*, 2012). These studies provide further evidence of the importance of amino acid metabolism for oocyte developmental competence.

Metabolic cooperativity between the oocyte and granulosa cell

The co-ordinated bidirectional communication between the developing follicle and its oocyte is required for the correct development of both compartments. This communication is mediated by extracellular factors and gap junctional coupling. Somatic cells support oocyte development by providing metabolites, and by regulating oocyte growth and maturation (Binelli and Murphy, 2010; Sutton-McDowall *et al.*, 2010). CC are also thought to aid fertilisation (Tanghe *et al.*, 2002). Thus, disruption of CC-oocyte communication before oocyte maturation or fertilisation decreases oocyte developmental competence (Zhang *et al.*, 1995). In turn, oocyte derived factors regulate GC/CC metabolism (Su *et al.*, 2009), theca and GC proliferation and differentiation, as well as cumulus expansion (Binelli and Murphy, 2010).

Homologous gap junctions between GC, and heterologous gap junctions between GC and the oocyte mediate their metabolic coupling by allowing the free transit of small molecules (<1kDa), including ions, amino acids, pyruvate, glucose, nucleotides and other signalling molecules (Su *et al.*, 2009; Wang *et al.*, 2012). Heterologous gap junctions have been described from the primordial follicle stage in mice (Mitchell and Burghardt, 1986) or the later secondary stage in cattle (Fair *et al.*, 1997). Their importance is evident from the fact that mice deficient for connexin 37 (the major connexin in heterologous gap junctions) are infertile and show follicles arrested at the late preantral stage, premature GC differentiation into luteal cells, and impairment of oocyte growth and meiotic competence (Carabatsos *et al.*, 2000). Similarly, mutation of the gene encoding for connexin 43 (the major connexin in homologous gap junctions) causes follicle arrest at the primary stage, as well as impairment of oocyte growth and meiotic competence (Ackert *et al.*, 2001). These studies seem to indicate that heterologous gap junctions are not essential for early follicle development, yet they appear key for normal oocyte growth and maturation.

Glucose is the preferred energy substrate for CC (Sutton-McDowall *et al.*, 2010), whereas oocytes of most mammalian species studied consume little glucose, pyruvate being the preferred energy substrate (Eppig, 1976; Rieger and Loskutoff, 1994). Glucose appears to be a more important substrate for oocytes of species such as pig and Rhessus monkey (Krisher *et al.*, 2007; Zheng *et al.*, 2007). In general, oocytes take up pyruvate efficiently, but have lower capacity for glucose transport, as well as limited expression and activity of some glycolytic enzymes (reviewed by (Purcell and Moley, 2009; Sutton-McDowall *et al.*, 2010)). Thus, oocytes rely on CC-derived oxidable substrates for synthesis of ATP, namely pyruvate (Biggers *et al.*, 1967; Johnson *et al.*, 2007; Harris *et al.*, 2009). ATP and glucose may also be directly transferred from GC/CC through gap junctions (Downs, 1995; Wang *et al.*, 2012).

Denuded oocytes also have a reduced capacity to take up some amino acids such as alanine, glycine, lysine and histidine. The uptake of such amino acids is enhanced by CC, which appear to pass them into the oocyte via gap junctions (Eppig *et al.*, 2005; Pelland *et al.*, 2009). Conversely, leucine uptake by murine oocytes appears inhibited by the presence of CC (Pelland *et al.*, 2009) (see table 1). When compared to bovine oocytes, CC also possess greater activity of the enzymes involved in amino acid metabolism such as aspartate aminotrasferase and particularly alanine aminotrasferase, as well as malate dehydrogenase (Cetica *et al.*, 2003). The reactions mediated by these enzymes produce substrates for the TCA cycle such as pyruvate, oxaloacetate and malate. Thus, CC could potentially provide the oocyte with not only amino acids, but also a range of intermediates for oxidative metabolism (Cetica *et al.*,

2003). Another example of cooperativity is the facilitative action that CC exert upon the synthesis and accumulation of GSH within the oocyte (de Matos *et al*., 1997). This action might be mediated by the capacity of CC to produce substrates for GSH synthesis such as cysteine (de Matos *et al*., 1997), or by direct gap junctional transfer of GSH to the oocyte (Mori *et al*., 2000).

Finally, Eppig *et al.,* (2005) reported that some paracrine signal from fully grown murine oocytes was able to up-regulate the expression of the solute carrier family 38, member 3 (SLC8A3, a sodium-coupled neutral amino transporter) in CC (Eppig *et al*., 2005). This transporter was expressed in CC but not in mural GC or oocytes, and showed preference for L-alanine and L-histidine. It appears, then, that oocytes can potentially increase the availability of such amino acids for gap junctional transfer (Eppig *et al*., 2005). Similarly, other experiments in mice have shown that key enzymes in the glycolysis (Sugiura *et al*., 2005) and cholesterol synthesis (Su *et al*., 2008) pathways are up-regulated in CC compared to mural GC, and that this expression pattern is controlled by oocyte derived factors. However, this phenomenon may be species-specific, as Sutton *et al.,* (2003) did not find an oocyte-mediated effect on bovine CC carbohydrate metabolism. Such differences could also be due to different methodologies and media composition, as Zuelke and Brackett (1992) previously found increased glycolytic activity in bovine COC but not if these cumulus complexes were oocytectomised.

Fatty acids as an endogenous energy source during oocyte maturation

After the breackdown of gap junctional contact following the preovulatrory surge of gonadotrophins, the final stages of oocyte maturation as well as fertilisation and early embryo development rely on the extracellular environment and endogenous substrates for energy homeostasis. Potential endogenous energy sources are glycogen, fatty acids and proteins. Mammalian oocytes and embryos contain relatively low levels of glycogen and lipids, except for farm species, which present characteristically large quantities of intracellular lipid, triglycerides being the major component (McEvoy *et al*., 2000; Sturmey *et al*., 2009). Glycogen accumulates during embryo cleavage and it is likely to be more important at the blastocyst stage (Flynn and Hillman, 1980; Ferguson and Leese, 2006). Proteins are unlikely to be used for ATP production, moreover glycogen and protein endogenous stores are insufficient to sustain development (Ferguson and Leese, 2006; Sturmey *et al*., 2009) and references therein). On the other hand, lipid droplets accumulate during oocyte growth, and fatty acid oxidation can generate high number of ATP molecules (around 106 ATP molecules from the complete oxidation of one molecule of palmitate), making these molecules good candidates for energy provision during oocyte maturation and early embryo development, especially in farm animals (Sturmey *et al*., 2009).

A number of studies have reported the association of mitochondria with lipid inclusions, vesicles and endoplasmic reticulum during oocyte maturation (Fair *et al*., 1997; Motta *et al*., 2000; Nagano *et al*., 2006; Sturmey *et al*., 2006). The association of mitochondria and lipid droplets is suggested to serve a source of fatty acids for mitochondrial oxidation (Nagano *et al*., 2006; Sturmey *et al*., 2006). Several lines of evidence support the idea that fatty acid oxidation is a source of ATP essential for oocyte maturation and beneficial during embryo development. For example, the triglyceride content of porcine and bovine oocytes decreases during IVM, as well as after fertilisation in the bovine (Ferguson and Leese, 1999; Sturmey and Leese, 2003). Mouse embryos have been shown to take up and oxidise radiolabeled palmitate (Flynn and Hillman, 1980). In addition, lipase activity increases in bovine oocytes following IVM (Cetica *et al*., 2002), and the expression of the rate-limiting enzyme of β-oxidation, carnitine palmitoyl transferase 1B (*Cpt1b),* is up-regulated in murine COCs following human chorionic gonadotropin (hCG)-induced ovulation (Dunning *et al*., 2010). Inhibition of β-oxidation during oocyte IVM blocked AMP-activated protein kinase-mediated meiotic resumption in the mouse (Downs *et al*., 2009) and impaired oocyte developmental competence in the mouse, pig and cow (Ferguson and Leese, 2006; Sturmey *et al*., 2006; Dunning *et al*., 2010). Moreover, inhibition of β-oxidation during embryo culture negatively affects blastocyst development in the mouse and cow (Hewitson *et al*., 1996; Ferguson and Leese, 2006; Dunning *et al*., 2010). It appears that mouse and porcine embryos can, to some extent, compensate for such inhibition by adjusting glucose metabolism (Hewitson *et al*., 1996; Sturmey and Leese, 2008). Finally, recent experiments have shown some improvement in embryo development following supplementation of murine and porcine IVM culture media (Dunning *et al*., 2010; Somfai *et al*., 2011) and bovine embryo culture media (Sutton-McDowall *et al*., 2012) with L-carnitine, a cofactor of Cpt1 present in plasma. The beneficial effect of L-carnitine could be due to its antiapoptotic action, its antioxidant action (with a concomitant increase of intracellular GSH in oocytes), and/or the up-regulation of β-oxidation (Dunning *et al*., 2010; Somfai *et al*., 2011; Sutton-McDowall *et al*., 2012). It is hypothesised that up-regulation of β-oxidation might result in increased availability of carbohydrates such as glucose for its use in other non-ATP producing pathways including synthesis of nucleic acids and hyaluronic acid, cell signalling (Sutton-McDowall *et al*., 2012) and redox regulation. This situation may also aid metabolic regulation and rapid cell proliferation in embryos by means of the Warburg Effect (Krisher and Prather, 2012; Redel *et al*., 2012). Yet, the culture of oocytes/embryos without external energy substrates only supports limited development (Downs and Hudson, 2000; Ferguson and Leese, 2006; Sutton-McDowall *et al*., 2012), indicating that endogenous stores might not to be sufficient to fully support oocyte and embryo developmental potential, especially in those species with little lipid stores such as the mouse.

Conclusion

Like in tumours, follicular metabolism is very diverse and is aimed at energy production and branched anabolism to support growth. At first sight, tumour metabolism is reminiscent of stem cell and their inner cell mass precursors, which display high glycolytic flux and low oxphos (the so called “Warburg effect”). Therefore, metabolism of the growing oocyte and early embryo might be different to tumour metabolism. However, *in vivo* metabolomic analyses suggest the existence of a continuum of bioenergetic remodelling in rat tumours according to tumour size and its rate of growth with both glycolytic tumours and oxidative tumours (Jose *et al*., 2011). Furthermore, the Warburg effect has been shown to be mediated by an embryonic isoform of pyruvate kinase (M2 isoform, the major isoform in mammalian oocytes and early embryos (Krisher and Prather, 2012), suggesting that specificities of tumour cells

might be operative in growing oocytes. The most striking similarity between tumour and oocyte metabolism is the importance of other, non-glucose-dependent, metabolic pathways such as fatty acid synthesis and catabolism (β-oxidation) and glutamine catabolism (glutaminolysis) (Biswas *et al.*, 2012; Jose *et al.*, 2011; Daye and Wellen, 2012). The metabolic pathways essential for both oocyte and tumour growth not only supply for energy demand, but also regulate the intracellular redox potential and provide reducing equivalent used for biosynthesis of lipids, proteins and RNAs. Therefore, one should consider oocyte metabolism not only for its impact on the energetic load but also on redox potential and anabolism.

The possibility to undertake the metabolic profiling of spent culture media by single oocytes/follicles combined with live imaging of the same oocyte/follicle, should make it possible to determine the metabolome of a healthy follicle for the first time. In addition, the emerging techniques provided by "the omics" (i.e. gene expression, metabolome and fluxome profiling) might open up new possibilities for the study of oocyte/follicle and embryo metabolism; giving it a multilevel, wider and more dynamic scope. Emphasising not only the uptake of substrates, but also the intermediary metabolism, metabolic pathways and fluxes, should be key for understanding normal follicle function and its regulation.

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