The importance of cumulus cells for oocyte maturation and embryo development

Het belang van cumuluscellen voor de eicelrijping en de ontwikkeling van embryo's

(met een samenvatting in het Nederlands)

Proefschrift

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General Introduction

In 1978, Louise Brown was the first child to be born with the help of *in vitro* fertilization (IVF) [1]. Today, this technique is widely used in both human clinical medicine and in a range of other mammalian species, for example cattle. In animals, the technique of *in vitro* embryo production is for example used to produce more progeny from a valuable female, while simultaneously allowing the use of different males; (this contrasts to multiple ovulation and embryo transfer where all embryos from a given cycle will have the same father). Although the number of *in vitro* produced embryos has risen over the years (Figure 1), the efficiency of IVF is far from optimal. This may have discouraged more widespread uptake of the technique and the contribution of *in vitro* produced bovine embryos to the total cattle ET sector in Europe averaged around 17.5% (±2 SD) in the years 2012 – 2016 [2–4]. Moreover, it is increasingly clear that there may be additional negative consequences of *in vitro* embryo production methods, in the form of epigenetic changes that could have lifelong effects on offspring health and susceptibility to certain diseases [5–7]. Indeed, a proportion of *in vitro* produced embryos develop into abnormal fetuses or suffer abnormal placental development [8], which can result in fetal death [9, 10]. Also in human clinical medicine, IVF is rather inefficient with only about 10-30% of embryos transferred into the uterus successfully implanting and giving rise to a live baby [11]. To improve the efficiency with which IVF embryos yield viable pregnancies and result in live birth, various methods to predict the developmental competence of a given oocyte or embryo have been tested; among other potential predictors, the gene expression profile of the surrounding cumulus cells have been examined for their ability to predict the ability of the enclosed oocyte to give rise to a live offspring [12].

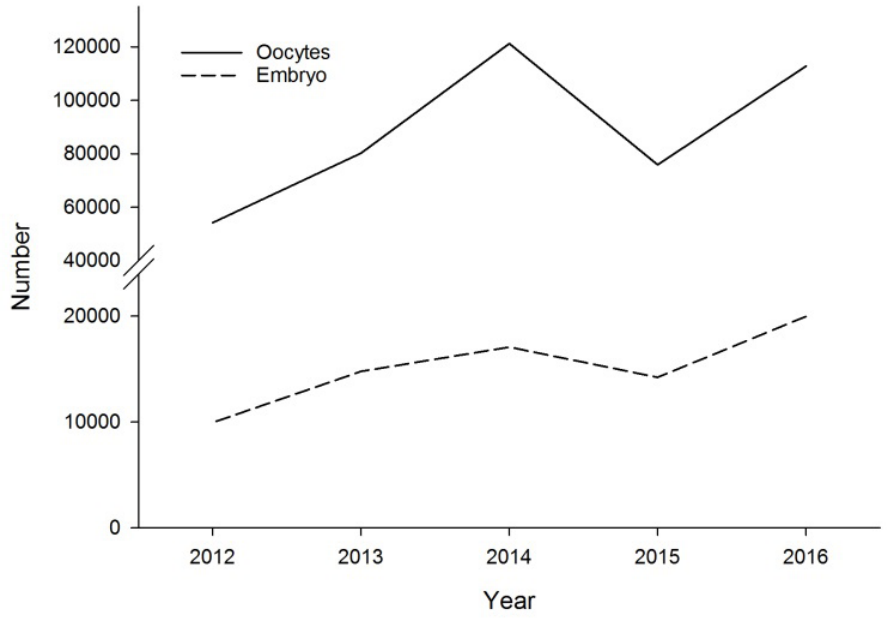

Figure 1: Number of oocytes obtained and *in vitro* produced bovine embryos reported in commercial programs in Europe. The solid line represents the number of oocytes collected for *in vitro* embryo production, the dashed line represents the number of *in vitro* produced embryos in Europe, data collected by the International Embryo Transfer Society [2–4].

At present, it is generally accepted that *in vitro* produced embryos are of inferior quality to *in vivo* embryos [13–16]. Importantly, the peri-conception environment of the oocyte in the follicle, and of the developing embryo in first the oviduct and then the uterus, have been shown to influence the epigenetic constitution of the developing embryo, and thereby the health of the resulting offspring. David Barker pioneered the concept that prenatal development can influence health throughout postnatal life. In particular, Barker and colleagues established that a low birth weight of babies is associated with a higher risk of developing type II diabetes or cardiovascular disease later in life [17]. These findings formed the basis for the Developmental Origins of Health and Disease (DOHaD) hypothesis, which has potentially profound implications for, and could yet influence the policies surrounding, artificial reproduction techniques [18]. In subsequent studies, the preimplantation period has been shown to be particularly vulnerable to disturbance and, in the case of IVF, the composition of the medium, or fluctuations in pH, temperature and oxygen tension have all been reported to influence the developing embryo [19–21]. A study in mice

showed that suboptimal IVF conditions affect postnatal growth and glucose tolerance, while even IVF and embryo culture conditions that were considered optimal resulted in hyperinsulinemia and increased fat accumulation in the resulting adult mice [22].

Although the fertilization of oocytes *in vitro* is an established technique, the maturation of oocytes *in vitro* has been a topic of critical debate because *in vitro* matured oocytes have a lower developmental competence than *in vivo* matured oocytes [23, 24]. This effect has also been described for human oocytes and, as a result, for human clinical programs only *in vivo* matured oocytes are collected, often after gonadotrophin stimulation to increase the number of mature follicles [25]. Indeed, in many countries including the Netherlands, *in vitro* maturation of oocytes for human IVF is not permitted. However, gonadotrophic stimulation also has negative side effects, for example it might influence implantation and the development of early pregnancy negatively [26] or it may increase chromosomal anomalies in preimplantation embryos [27]. It therefore may be sensible to reconsider the use of *in vitro* maturation to obtain developmentally competent oocytes. To date, however, it is not known how to reliably identify a developmentally competent oocyte, neither is it understood which processes are important for optimal oocyte maturation. Moreover, the exact roles of cumulus cells during oocyte maturation, and their influence on the developmental competence of the oocyte are not well understood. Since this type of research cannot be performed using human oocytes, animal models can be important in generating the information necessary to optimize oocyte maturation. A good animal model to investigate oocyte maturation, or the influence of the cumulus cells on oocyte developmental competence, is therefore required. The cow has several useful characteristics as a model for human reproduction, compared to the more widely used model species such as the mouse and rat. For example, like man the cow is a mono-ovulatory species, and the time course of both folliculogenesis and oocyte maturation are similar in these two species [28, 29]. A further advantage of the cow, is the availability of ovaries, and therefore oocytes, from slaughterhouses.

Folliculogenesis and oogenesis

During mammalian female embryo development, approximately three weeks after fertilization, the primordial germ cells develop in the yolk sac epithelium close to the developing allantois [30]. These cells subsequently migrate via the hindgut mesentery towards the genital ridge, while proliferating by mitosis. After arriving at the developing gonadal ridges, the cells are called oogonia.

Within the gonads, mesenchymal cells condense around the oogonia and, together, they form the primordial follicles. At this point, mitotic division stops, the oogonia embark on meiosis, and they are now known as primary oocytes. These oocytes arrest at the diplotene stage of the first meiotic division, and the chromosomes become decondensed and localized in a nucleus-like structure known as the germinal vesicle (GV). In man and cow, the formation of primordial follicles occurs before birth; in species like mice or rats, primordial follicle development occurs after birth [31, 32].

Following puberty, development of some primary follicles occurs in waves, with the granulosa cells within primary developing follicles changing from a flat morphology to a cuboidal shape, accompanied by proliferation to form several layers of granulosa cells; this follicle is now termed a secondary follicle. Until the secondary follicle stage, oocyte and follicle development is gonadotropin independent. From the secondary follicle stage, the granulosa cells start to develop follicle stimulating hormone (FSH) receptors, whereas the theca cells which surround the basal lamina develop luteinizing hormone (LH) receptors. These receptors are required for the gonadotropin-dependent stages of follicle development. At this stage, the oocyte begins to accumulate lipids, proteins and mRNA for later development, and to form a glycoprotein capsule, the zona pellucida, between the oolemma and the surrounding granulosa cells. The granulosa cells in direct contact with the zona pellucida form transzonal cytoplasmic projections that contact to the oocyte via gap junctions; these gap junctions are important for the exchange of small molecules between the oocyte and the surrounding cells [33, 34].

The next stage of follicular development is formation of a fluid filled cavity known as the antrum, to yield an antral follicle. The development from primordial to antral follicle takes several months in man and cows and only starts at puberty, when the levels of FSH and LH in the blood increase. A proportion of the developing follicles become FSH responsive and continue to grow, whereas other follicles that developed contemporaneously fail to develop gonadotropin receptors and instead undergo atresia. The growing follicles then pass through a second selection process during which one (e.g. human, cow) or more (e.g. mouse, pig) make a switch from FSH to LH-responsiveness and develop to the preovulatory stage, whereas the other follicles become atretic [32, 35, 36]. During antral follicle development, the granulosa cells start to differentiate into two different subtypes, the cumulus cells, which enclose the oocyte and the mural granulosa cells, which line the

antrum of the follicle [37, 38]. In the growing follicle, shortly before ovulation and triggered by an LH surge, the oocyte starts to mature and resumes meiosis.

Maturation of an oocyte is characterized by nuclear maturation, resulting in the extrusion of the first polar body and arrest of the oocyte in metaphase of the second meiotic division (MII), cytoplasmic maturation and expansion of the cumulus cells. The differentiation of cumulus cells from granulosa cells is directed by oocyte-secreted factors, including growth differentiation factor-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15) [39, 40]. The expanded cumulus cell matrix consists predominantly of hyaluronic acid. Ovulation occurs in response to an LH surge which triggers meiotic and cytoplasmic maturation of the oocyte [33]. Figure 2 shows a schematic overview of folliculogenesis.

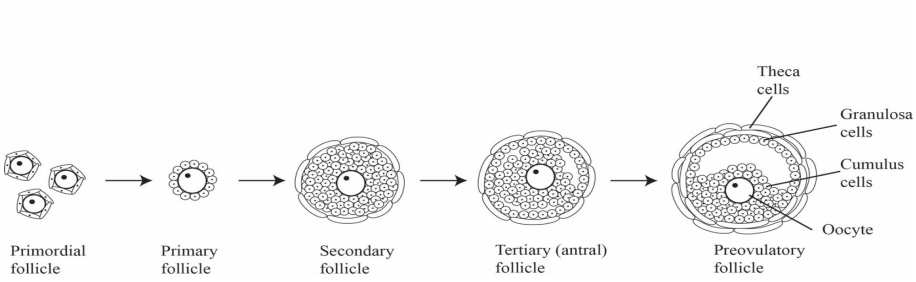

Figure 2: Schematic overview of folliculogenesis
Development and growth of the follicle and oocyte in mammals.

Impact on cumulus cells on oocyte developmental competence

Cumulus cells are specialized cells that evolve from granulosa cells. Two different granulosa cell species can be distinguished: the mural granulosa cells, which line the follicular wall and have a primarily steroidogenic role, and the cumulus cells which surround the oocyte [38]. Mouse studies have demonstrated that cumulus cells are important for ovulation and the acquisition of oocyte developmental competence [41]; indeed removal of cumulus cells before maturation leads to impaired fertilization and blastocyst development [42]. Cumulus cells are connected to the oocyte through gap junctions [36], transmembrane structures consisting of six connexin proteins. The most important components of the gap junction proteins between oocyte and cumulus cells are connexin-37 and connexin-43 [43]. Indeed, a mutation in connexin-37 has been reported to lead to sterility in female mice [44].

The importance of cumulus cells has been shown for various developmental steps in the oocyte, including cytoplasmic maturation, which is also required for the formation of the pronucleus [45]. In addition, the percentage of human *in vitro* embryos graded as 'good' after intracytoplasmic sperm injection (ICSI) was reportedly increased if some cumulus cells remained attached to the oocyte, compared to completely denuded oocytes [46]. The oocyte is dependent on energy substrates, such as glucose, but has itself a poor capacity to utilize glucose [47, 48]. The cumulus cells aid in the conversion of glucose into pyruvate, the preferred energy substrate of the oocyte. Glucose is also used for other important processes, including nucleic acid and purine synthesis or mucification of the extracellular matrix and cellular homeostasis [48, 49].

Appropriate expansion of the cumulus investment, which is triggered by the oocyte secreted factors GDF-9 and BMP-15, is also critical for fertility. GDF-9 and BMP-15 stimulate genes including *hyaluronan synthase 2* (*HAS2*), *tumor necrosis factor-induced protein-6* (*TNFIP6*), *pentraxin 3* (*PTX3*), *prostaglandin synthase 2* (*PTGS2*) and *gremlin 1* (*GREM1*) [50–53]. Indeed, knockout mice lacking *Ptx3* or *Tnfip6* are sub-fertile or sterile [52, 54, 55]. These genes are not only involved in cumulus expansion, but are also involved in glycolysis, stimulation of sterol biosynthesis and amino acid transport from the cumulus cells to the oocyte [51–53, 56]. Furthermore, a study on bovine cumulus cells showed a gradient of BMP-6 and BMP-15, factors known to prevent apoptosis, from the inner corona cells to the outer cumulus cells. This BMP gradient was inversely correlated to cumulus cell apoptosis, which was higher in the outer cells than the inner cumulus cells [57].

During cumulus cell expansion an extracellular matrix is formed, predominantly from hyaluronic acid secretion by the cumulus cells [58]. Hyaluronic acid is encoded by *HAS2*, the expression of which has been proposed as a marker for embryo quality [12]. This hyaluronic acid is anchored to the cumulus cells by the cell surface protein CD44 [59, 60]. Other important components of the extracellular matrix include proteoglycans (e.g. versican) that bind to hyaluronic acid and stabilize the extracellular matrix by cross-linking the hyaluronic acid molecules [61].

During assisted reproductive technologies, such as ICSI, the cumulus cells are removed from the oocytes. Indeed, removing some cumulus cells before IVF is not harmful to later embryo development. Given the integral role of cumulus cells in oocyte development, they could represent a good non-invasive source of material for the prediction of oocyte developmental competence. Indeed,

expression of specific mRNA species in cumulus cells has been reported to correlate with the developmental competence of the oocyte. For example, *HAS2*, *PTGS2*, *CD44* and *TNFAIP6* expression were positively associated with oocyte developmental competence [12, 62]. However, to date none of these proposed markers have proven to be able to prospectively predict the developmental competence of oocytes, where differences in methodology in the various studies may in part explain discrepancies between cumulus transcriptomes.

MicroRNAs

Cells can regulate their gene expression in various different ways; one of these is post-transcriptional regulation via microRNAs (miRNAs). MiRNAs are small (20-23 nucleotides), non-coding, single-stranded RNA molecules [63]. By binding to the 3' untranslated region (UTR) of their target mRNA, miRNAs can regulate post-transcriptional gene processing. Three different mechanisms by which miRNAs can exert their regulatory functions have been suggested: 1) translational inhibition, 2) translational enhancement, and 3) mRNA degradation [64].

The processing of miRNAs starts in the nucleus where transcription either via RNA polymerase II or polymerase III yields primary miRNA transcripts (pri-miRNA) [65, 66]. For the next miRNA processing step, the enzymes DROSHA and DiGeorge critical region 8 (DGCR8) form a complex to perform the endonucleolytic cleavage that allows the precursor-microRNAs (pre-microRNAs) to emerge [67]. For further processing, the pre-microRNA is transported from the cell nucleus to the cytoplasm by a complex consisting of Exportin-5 and Ran-GTP [68]; only correctly processed pre-miRNAs are transported into the cytoplasm, which makes this step a quality control point [69]. The next processing steps occur in the cytoplasm where DICER, an RNase III enzyme, forms a complex with Tar RNA binding protein (TRBP). TRBP is responsible for the stability of DICER and facilitates the cleavage process [70]. After the pre-microRNA arrives in the cytoplasm, it is cleaved by DICER into a double stranded product with a length of approximately 22 nucleotides. Subsequently, the functional strand of the microRNA duplex is loaded, with the help of the Argonaute-2 (AGO-2) protein, into the RISC loading complex (RLC). This complex consists of DICER, TRBP, PACT and AGO-2 and, together with the single-stranded miRNA, they form the RNA-inducing silencing complex (RISC) [70, 71]. The decision as to which strand is loaded into the RISC is made by thermodynamic stability, the strand

with the less stable 5' base pair is loaded into the RISC [72], whereas the other strand is degraded. The RISC is thought to be able to regulate mRNA expression in three different ways, namely cleavage, translational repression or deadenylation. Figure 3 depicts the miRNA processing pathway.

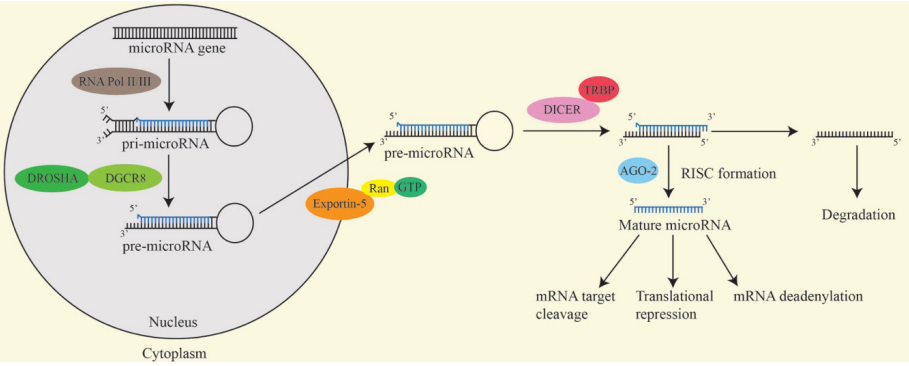

Figure 3: microRNA processing pathway

After transcription, genomically encoded miRNAs are cleaved by RNA Polymerase II/III to generate the primary microRNA (pri-microRNA). The DROSHA-DGCR8 complex then cleaves this primary miRNA to yield the precursor-microRNA (pre-microRNA). This hairpin structure is exported into the cytoplasm by Exportin-5-Ran-GTP, where it is further processed by a DICER-TRBP complex to yield a double strand of the mature length. The passenger strand is degraded, while the functional strand is loaded into the RNA-induced silencing complex (RISC), together with the Argonaute-2 protein (AGO-2). The RISC is guided to the target mRNA, with expression regulated by cleavage, translational repression or deadenylation.

miRNAs in the cumulus-oocyte complex

Post-transcriptional regulation of genes is very important during oocyte development because the genome is transcriptionally silent from the GVBD stage until zygotic activation [73]. Various miRNA species have been identified within the reproductive tract (reviewed in [64]), but their functions remain largely unknown. It seems however that miRNAs are important for oogenesis and embryo development. In mice, genetic loss of *Dicer* and *Ago-2* leads to abnormal oogenesis [74, 75], while *Dicer1* knockout mice show embryonic lethality at embryonic day 11.5 [76]. MiRNAs have also been identified in granulosa cells; for example, inhibition of miR-21 activity is reported to lead to increased apoptosis and to suppress ovulation [77]. In several species, including man and cattle, miRNAs have been shown to be important during oocyte maturation and embryo development [78–80]. During oocyte maturation, the expression of miRNAs changes [75, 81]. In addition, the miRNA profile in oocytes changes during ageing, which may contribute to reduced oocyte developmental competence [82, 83]. In a previous study in cattle oocytes, it was shown that miR-21 and miR-130a expression correlates positively with successful development from the GV stage to an 8-cell embryo, although the exact mechanisms responsible for these observations were not examined [84].

Expression of miRNAs has also been detected in cumulus cells. A comparison of the number of RNA sequences in cumulus cells versus MII oocytes showed more reads in the cumulus cell complex [79]. It is also possible that miRNAs from the cumulus cells are transported to the oocyte via gap junctions in the transzonal processes. Indeed, mRNAs that are highly expressed in oocytes have been shown to be potential targets of miRNAs expressed in cumulus cells [79]. Several studies have suggested that miRNAs expressed in cumulus cells could regulate mRNAs involved in the formation of the extracellular matrix. For example, it was reported that miR-224 compromises cumulus expansion by targeting *Ptx3* [85], a gene critical for cumulus expansion [55] and stability of the extracellular matrix [52]. In porcine cumulus cells, overexpression of miR-378 inhibited cumulus expansion, possibly by downregulating *HAS2* and *PTGS2* expression [86]. In man, the miRNA expression profile of corona radiata cells differs from that of the more distant cumulus cells and gene ontology analysis indicated that the miRNAs in corona cells are associated with glycolysis and amino acid metabolism [87], thereby hinting at a role of corona radiata derived miRNAs in regulating metabolic activity in and around the oocyte. Since miRNAs appear to influence cumulus expansion and metabolic processes during oocyte maturation, it is possible that miRNA expression in cumulus cells could help predict oocyte developmental competence.

Metabolomics

In living cells, metabolism is vital for two processes: 1) providing energy to maintain cellular homeostasis and support specialized functions, 2) provision of metabolites for biosynthesis of cellular components [88]. Metabolites are the result of all transcriptomic and proteomic processes in a cell and they are representative of proper or disturbed cellular function. Living cells, including those in COCs and embryos, secrete metabolites into their environment that reflect their metabolic processes. By analysing these metabolites, information about the health of a COC/embryo can be generated. In the past, it was common to analyse changes in concentrations of single metabolites, such as pyruvate uptake [89] or amino acid turnover [90]; such analysis was limited to a single pathway. More recently, metabolic profiling has developed as a way to assess different pathways in cells simultaneously, and thereby provide a much more complete view of the processes in the cells from which more meaningful and powerful conclusions about the health of cells can be drawn. However, there are some difficulties with metabolic profiling, in that some metabolites are unstable and different molecules have different chemical properties, this needs to be taken into account when designing methods and interpreting the outcome of metabolic screening [91].

It has been proposed that germ cells are in a 'quiet' metabolic state with limited mitochondrial activity and limited formation of reactive oxygen species, to protect the mitochondrial DNA (mtDNA). During oxidative phosphorylation, reactive oxygen species are generated, which can destabilize and damage the mtDNA [92]. Apart from oxidative phosphorylation, reactive oxygen species can also be generated by proteins involved in glycolysis, β-oxidation and the tricarboxylic acid cycle. One of these enzymes is pyruvate dehydrogenase [93], which links glycolysis with the tricarboxylic acid cycle by converting pyruvate into acetyl-CoA. Another potential ROS producer is aconitase [94], which catalyses the conversion of citrate to isocitrate, and *vice versa*, within the tricarboxylic acid cycle. If cumulus cells are able to take over some of these metabolic processes, it might help reduce the reactive oxygen species production within the oocyte.

Even if the oocyte is in a 'quiet' metabolic state, energy is required for the resumption of meiosis and for growth [47, 95]. An important energy substrate during oocyte maturation is pyruvate, which is derived from glucose during glycolysis. Since the oocyte utilizes glucose poorly, it is dependent on the cumulus cells to metabolize glucose to pyruvate [96]. The poor glucose utilization of oocytes can be explained by a low glycolytic activity, as a

result of low activity of phosphofructokinase [97], the rate-limiting enzyme in glycolysis. In the oocyte, pyruvate and lactate are used for energy production; they are metabolised via the tricarboxylic acid cycle, followed by oxidative phosphorylation to produce ATP [98].

Glycolysis is not the only metabolic pathway used to generate energy; indeed, lipid metabolism can provide more energy (ATP) than glycolysis. For example, the oxidation of palmitate generates 106 molecules of ATP, compared to only 30 molecules generated by glucose oxidation. Fatty acids are stored intracellularly in lipid droplets within the oocyte and cumulus cells [99, 100]. The cumulus cells seem to regulate the uptake of fatty acids [101], but also protect the oocyte against the negative effects of saturated free fatty acids [102]. During maturation, the oocyte is a dynamic cell with a high demand for energy to facilitate nuclear maturation and processes such as organelle redistribution and the storage of maternal RNA and metabolites [103]. The energy from fatty acids is generated through mitochondrial β-oxidation, where carnitine is an important cofactor involved in transporting free fatty acids into the mitochondrion [104].

Amino acids are also important for oocyte maturation and are taken up via specialized transporters, where different amino acid transporters have their own specific range of potential cargo. An increased uptake of amino acids has been reported in oocytes enclosed by cumulus cells, these included glycine, alanine, proline, histidine and serine [105–107]. Alanine and histidine are substrates for the amino acid transporter SLC38A3; mRNA for this transporter has been found in cumulus cells but not in the oocyte. This lack of amino acid transporter expression in oocytes leads to a poor uptake of amino acids, this was also shown by a higher histidine and alanine accumulation in oocytes with an intact cumulus complex compared to denuded oocytes [106]. Moreover, amino acid supplementation during *in vitro* maturation has a positive effect on the maturation of denuded oocytes [108] and on subsequent embryo development [109]. To find a marker to predict IVF outcome, amino acid turnover in spent embryo culture medium has been examined, and it was reported that asparagine, glycine and leucine concentrations correlate with pregnancy and live birth [90]. These findings were only in partial agreement with the amino acid turnover that predicted blastocyst development for *in vitro* cultured embryos [110]. The analysis of single metabolites or single pathways considers only a part of the overall process occurring during oocyte maturation or embryo development. Analysis of the entire metabolomic profile might be more helpful in understanding the processes occurring during oocyte maturation or embryo development.

Moreover, pathways and metabolites critical to proper oocyte maturation might be identified and inform improvements in conditions for the *in vitro* maturation of oocytes in the future.

Glucose alters the metabolomic profile of embryos

Maternal hyperglycemia induced by type I or type II diabetes, obesity or an unsuitable diet results in a higher risk of miscarriage and an increased risk of fetal congenital abnormalities [111]. In addition, it has been established that maternal health during pregnancy affects the long-term health of the offspring [112]. Indeed, in a diabetic mouse model it has been established that oocytes are smaller and show delayed meiotic maturation compared to oocytes from non-diabetic mice [113, 114]. Similarly, in diabetic rats, blastocysts have a reduced number of inner cell mass cells, and a higher percentage of embryos exhibit fragmentation [115, 116]. High maternal glucose concentrations affect not only embryo morphology, but also metabolic pathways. It has been demonstrated that polyol and hexosamine pathway activity are increased by diabetes, as is the formation of advanced glycation end-products (AGEs) [117]. An increase in AGEs leads to cell damage characterized by increased reactive oxygen species production and, therefore, elevated oxidative stress [118]. The polyol pathway oxidizes fructose to sorbitol, catalysed by sorbitol dehydrogenase, and accompanied by a reduction of NAD^+ to NADH. As a consequence the NADH:NAD^+ ratio increases in the cell, leading to an inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity and an increase in the concentration of the AGE precursor, glyoxal [119]. It has also been demonstrated that increased sorbitol production during embryo culture leads to a delay in blastocyst development [120].

A further characteristic of high glucose levels is increased flux through the hexosamine pathway which converts fructose-6-phosphate into glucosamine-6-phosphate, followed by conjugation to UDP-N-acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc is involved in the formation of *O*-linked glycoproteins, and this *O*-linked glycosylation has an embryotoxic effect [121].

Finally, fatty acid uptake and β-oxidation change under diabetic conditions. In blastocysts from diabetic rabbits, a higher content of lipid droplets in the embryoblast and trophoblast were detected [122]. Adiponectin was proposed to play a central role in this change, because it stimulates glucose uptake and its expression increases under diabetic conditions [118, 122].

The number of women of reproductive age with metabolic disorders, such as diabetes, is increasing. However, the processes that are altered in early embryos exposed to these suboptimal environments are poorly understood, metabolic profiling of these embryos could help us to understand how metabolic processes are altered in these embryos and what the consequences are likely to be for pregnancy and epigenetic programming.

Thesis outline

In this thesis, the role of cumulus cells during maturation and the acquisition of developmental competence was investigated. **Chapter 2** describes the sequencing of miRNAs to identify miRNAs expressed in cumulus cells that could help predict the developmental potential of the enclosed oocyte. Since no overall expression differences were found, **chapter 3** investigated how miRNA expression levels change over the course of maturation, and how they differ between cells from different parts of the cumulus complex.

Chapter 4 describes how cumulus cell contact and COC-conditioned maturation medium influence the ability of oocytes to develop into blastocysts after fertilization. In addition, the metabolomic profile of cumulus cells and the medium in which they were incubated during maturation was analysed to identify metabolites that may influence the success of maturation.

One of the most prevalent metabolic disorders worldwide is diabetes, which is characterized by resting hyperglycaemia. In **chapter 5**, we investigated how high glucose concentrations during fertilization and embryo development can influence the metabolism and the quality or health of a blastocyst.

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MicroRNA expression in bovine cumulus cells in relation to oocyte quality

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Abstract

Cumulus cells play an essential role during oocyte maturation and the acquisition of fertilizability and developmental competence. Micro(mi)RNAs can post-transcriptionally regulate mRNA expression, and we hypothesized that miRNA profiles in cumulus cells could serve as an indicator of oocyte quality. Cumulus cell biopsies from cumulus-oocyte-complexes that either yielded a blastocyst or failed to cleave after exposure to sperm cells were analysed for miRNA expression. On average, 332 miRNA species with more than 10 reads and 240 miRNA species with more than 50 reads were identified in cumulus cells; this included 9 previously undescribed microRNAs. The most highly expressed miRNAs in cumulus cells were miR-21, members of the let-7 family and miR-155. However, no repeatable differences in miRNA expression between the cumulus cells from oocytes that became blastocysts versus those from non-cleaved oocytes were identified. Further examination of individual cumulus cell samples showed a wide variability in miRNA expression level. We therefore conclude that miRNA expression in cumulus cells cannot be used as an oocyte quality marker.

Introduction

Mammalian oocytes acquire the capacity to become fertilized and developmental competence during their final maturation within the ovary. These oocytes originate from female primordial germ cells that proliferated while migrating towards the gonadal ridges, and continue to proliferate until gonadal sex differentiation. The cells are classified as oocytes from the moment they stop dividing and enter meiosis [1]. Meiosis in developing oocytes arrests at the first prophase and, depending on the species, this meiotic arrest can be maintained for decades [2]. As early as this time point the oocyte acquires competence to develop into an embryo after fertilization [3]. The oocyte only resumes meiosis shortly before ovulation, in response to luteinizing hormone stimulation. After the resumption of meiosis, and in addition to chromosome segregation, the oocyte needs to redistribute organelles and proteins in a process known as cytoplasmic maturation [4]. For these processes, the oocyte relies on molecules present within the follicular fluid and on intimate contact with the somatic cells that surround the oocyte, known as cumulus cells. Together, they form the cumulus-oocyte-complex (COC). Tightly regulated communication between the oocyte and surrounding cumulus cells is important at various stages, including oocyte growth, maturation, and fertilization [5–7]. Exactly how the cumulus cells contribute to the fertilizability and developmental competence of the oocyte, hereafter referred to as oocyte quality, is not known. From in vitro maturation and fertilization experiments, it has however become clear that large differences exist between the quality of oocytes, and it seems likely that cumulus cells are at least partly responsible for the oocyte quality.

Cumulus cells are connected to each other and to the oocyte by direct cell-cell contact. Exchange of small (<1kDa) molecules, like cAMP, is thought to occur through gap junctions [8–10] whereas passage of larger molecules, including RNA, can occur via zonula-adherens-like junctions [11, 12]. Communication between the oocyte and cumulus cells is bidirectional [13], with the cumulus cells providing the oocyte with factors and signaling molecules [6], but the oocyte also sending signals to the cumulus cells [9]. If communication between the cumulus cells and the oocyte is disturbed, oocyte quality can be affected which can in turn compromise subsequent embryo development [14]. Indeed, *in vitro* maturation of oocytes in the absence of cumulus cells markedly reduces the developmental competence of oocytes [15].

For human in vitro fertilization (IVF) in particular, it would be beneficial if fertilizable and developmentally competent oocytes could be identified before fertilization, without damaging the oocyte. Oocyte morphology has demonstrated to be a poor predictor of quality and is rather subjective [16, 17]. A molecular marker to predict oocyte quality before fertilization would improve embryo development and pregnancy rates, and could be used to reduce the number of surplus embryos produced and the resulting ethical conflict regarding the ultimate fate of these embryos [18]. One useful model animal for human oocyte development is the cow since, like man, it is a mono ovulatory species; in addition, it has a comparable time course of oogenesis [19, 20]. Furthermore, the blastocyst development rate after in vitro fertilization in cattle is ~35%, comparable with that obtained in human IVF programs [21, 22]. Not surprisingly, efforts have been undertaken to identify non-invasive quality markers for oocytes, including analysis of gene expression in cumulus cells [23–27] and investigation of the composition of follicular fluid [25, 28].

MicroRNAs (miRNAs) are highly conserved, small (21-25 nucleotides in length), non-coding RNAs that can regulate gene expression [29, 30]. Via sequence complementarity, the seed sequences of miRNAs can bind to the 3'UTR of target mRNAs and, in combination with an Argonaute family protein, the miRNA can regulate the expression of the target gene by degrading that mRNA or inhibiting its translation. It has been suggested that a single miRNA species can regulate ~100 different target genes [31], enabling a broad level of regulation.

MiRNAs are processed from longer transcripts by the RNase III-like enzymes Dicer and Drosha. Genetic deletion of Dicer results in embryonic lethality in mice [32], and when Dicer was specifically deleted in growing oocytes, they failed to cleave after fertilization, indicating the importance of miRNAs to oocyte competence [32]. Apart from expression in oocytes, miRNAs have also been detected in cumulus cells [14, 33] and it has been demonstrated that the miRNA expression profiles of both oocyte and cumulus cells change during maturation [33–37]. Identification of differentially expressed miRNAs in corona radiata cells and the outer cumulus cells from human oocytes indicated a role for these miRNAs in nutritional and regulatory signaling between oocytes and cumulus cells [38].

In this study, we examined the miRNA expression profiles of bovine cumulus cells and compared those from COCs that yielded blastocyst after fertilization versus those that remained non-cleaved after exposure to sperm.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) unless otherwise indicated.

Collection of COCs, *in vitro* maturation and fertilization

Cumulus-oocyte-complexes were collected and cultured as described previously [46]. COCs were matured for 23 hours in groups of 60–70 in $NaHCO_3$-buffered M199 (Gibco BRL, Paisley, UK) supplemented with 10% fetal bovine serum, 1% Penicillin-Streptomycin (Gibco BRL), and 0.05 IU follicle stimulating hormone/ml (Organon, Oss, The Netherlands) at 39°C in a humidified atmosphere of 5% CO_2-in-air. After maturation small pieces of the cumulus complexes were retrieved using a narrow-bore Pasteur pipette. These cell clumps were transferred to lysis buffer (Exiqon, Vedbaek, Denmark) containing 10 µl/ml β-mercaptoethanol, labelled and stored at -80°C until further use. Subsequent individual fertilization and embryo culture enabled retrospective linking of the cumulus pieces to the quality of the oocyte that they had enclosed.

In vitro fertilization was performed as described previously [47]. In short, percoll-washed sperm cells from a bull of proven fertility were added to fertilization medium supplemented with 1.8 IU/ml heparin, 20 µM d-penicillamine, 10 µM hypotaurine, and 1 µM epinephrine at a final concentration of 1×10^6 sperm cells/ml. Terasaki microwell plates (Nalge Nunc International, Rochester, NY, USA) were used to allow single oocyte fertilization, and one oocyte per well was incubated in 10 µl of fertilization medium and covered with light mineral oil (Irvine Scientific, Santa Ana, CA, USA). After 18–22 hrs at 39°C in a humidified atmosphere containing 5% CO_2 and 7% O_2, presumptive zygotes from single oocyte fertilization were denuded by repeated aspiration through a narrow pipette; presumptive zygotes from group culture were denuded by vortexing for 3 min; all denuded zygotes were transferred to synthetic oviductal fluid (SOF) for further culture [46]. On day 5 of culture, cleaved embryos were transferred to a new well containing fresh SOF, and cultured until day 8.

RNA isolation

Total RNA was isolated using the miCURY RNA isolation kit (Exiqon), according to the manufacturer's protocol. In short, samples were lysed with 350 µl lysis buffer containing 10 µl/ml β-mercaptoethanol, and shearing stress

was applied by aspiration through a 23-gauge needle, followed by the addition of 200 µl 100% ethanol. The samples were loaded onto the columns provided, washed by centrifugation, and the RNA was eluted using 50 µl elution buffer. An additional concentration step was performed on cumulus cell samples from individual COCs. To do this, the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) was used and the RNA was isolated as per the manufacturer's instructions. In brief, 350 µl RLT buffer and 675 µl 100% ethanol were added to 100 µl of sample. This solution was transferred to an RNeasy MinElute spin column and, after three centrifugation and washing steps, the RNA was eluted using 17 µl RNase-free water.

Library preparation and Next Generation sequencing

Library preparation and next generation sequencing was performed by Exiqon. The concentration of total RNA from pooled cumulus samples (10–16 COCs) varied between 10- and 20 ng/µl. To obtain a minimum of 100 ng total RNA, 45 µl of each sample was converted into miRNA next generation sequencing libraries using a NEBNEXT kit (New England Biolabs, Ipswich, MA, USA) as per the manufacturer's instructions. Adaptors were ligated to the ends of the sequences and libraries were generated using 3' and 5'rapid amplification of cDNA ends (RACE) like protocols. Libraries were purified after 15-cycle pre PCR using QiaQuick columns (Qiagen) and size fractioned on a LabChip XT (Caliper, Hopkinton, MA, USA). Bands representing 15-40 bp fragments were excised with an automated gel cutter, evaluated using a Bioanalyzer DNA 2100 chip and quantified by qPCR. Samples were normalized and pooled in equimolar concentrations [48] and sequenced using v3 sequencing (Illumina Next Seq 500 system, San Diego, CA, USA). Eight-level quality score-binning was used, enabling a more compact storage of raw sequences [49].

The miRNA sequencing data have been deposited in NCBI's Gene Expression Omnibus database and are accessible through Geo series accession number GSE94771.

First-strand synthesis and qRT-PCR

The universal cDNA synthesis kit II (Exiqon) was used to perform first-strand synthesis, with 10 µl template RNA in a total volume of 20 µl as per the manufacturer's instructions. Samples were incubated for 1 h at 42°C followed by 5 min at 95°C, and then immediately cooled on ice.

PCR was performed in a total volume of 15 µl with 1 µl cDNA, 1.5 µl locked nucleic acid (LNA) primer pair (Exiqon), and 7.5 µl PCR master mix (BioRad, Hercules, CA, USA). Quantitative real time PCR was performed using a CFX Connect™ Real-Time PCR detection system (Bio-Rad). The program started with 10 min at 95°C followed by 40 cycles each of 10 sec at 95°C and 60 sec at 60°C. Melting curves were plotted after each cycle series. A standard curve of a 3-fold dilution series was obtained by plotting the log of the starting amount against the cycle threshold value of the dilution series. Different potential reference miRNAs were tested and, in accordance with GeNorm guidelines [50], the three small RNAs with the most stable M-Value were considered optimal and adequate for normalization (Supplementary Figure 1). The geometric means of expression for the reference miRNAs miR-26a, miR-191 and let-7a were used for normalisation of the target miRNAs.

Statistical Analysis

Comparisons between culture conditions and the resulting cleavage and blastocyst production rates were performed using Students t-tests. Ct-values of possible reference miRNAs were analysed using GeNorm [50].

Results

Comparison of individual versus group culture

In order to identify miRNA species expressed in cumulus cells that could potentially predict quality of the enclosed oocyte, cumulus biopsies were obtained from cumulus-oocyte complexes (COCs) after in vitro maturation. Subsequent individual fertilization of COCs and individual embryo culture were performed to follow the progression of each oocyte individually and thereby allow correlation of the cumulus miRNA expression profile to oocyte quality, retrospectively. First, to analyse whether individual embryo culture affected the efficiency of blastocyst formation, embryos were cultured either individually but with shared culture medium, or in groups of 30–50. The percentages of oocytes that cleaved or developed into blastocysts were similar for the two culture conditions (Table1), demonstrating the validity of the single embryo culture system.

			Day 5			Day 8
Culture type	**Total**		**Not cleaved**	**2 - 8**	**>8**	**blastocyst**
Group	481	n:	121	207	153	129
		%:	25.2	43	31.8	26.8
Indivudual	188	n:	35	86	67	53
		%:	18.6	45.7	35.6	28.2

Table 1: Comparison of group and individual embryo culture
Comparison of cleavage and further development of bovine COCs/zygotes cultured in groups or individually.

Sequencing of miRNAs in cumulus cells

In order to determine whether the miRNA expression profiles of cumulus biopsies from COCs that contained fertilizable and developmentally competent oocytes (i.e. yielded a blastocyst) differed from those that contained oocytes of poor quality, cells were harvested for small RNA sequencing. After COCs had been matured, a biopsy of the cumulus cells was obtained and the COCs were cultured individually for fertilization and embryo development.

Cumulus cells surrounding an oocyte that (1) developed into a blastocyst or (2) failed to cleave after exposure to sperm were pooled to ensure sufficient amounts of RNA (each pooled sample contained cumulus cells from 10-16 individual COCs). Three pooled samples of cumulus cells harvested from oocytes that did not cleave (referred to as A, B and C) and three pooled samples of cumulus cells that had enclosed oocytes that developed into blastocysts (D, E and F) were sequenced for small RNAs; they yielded an average of 14 million reads (Figure 1).

MicroRNAs were identified according to entries in miRBase release 20. A mean of 332 miRNA species had more than 10 reads (Figure 2, blue bars), while 240 miRNA species had more than 50 reads per sample (Figure 2, red bars). The majority of the miRNAs was present in all samples; of the miRNA species identified, 276 with more than 10 counts and 205 with more than 50 counts, were present in all samples. No repeatable differences in the numbers of miRNAs identified were present between the two types of sample (Figure 2).

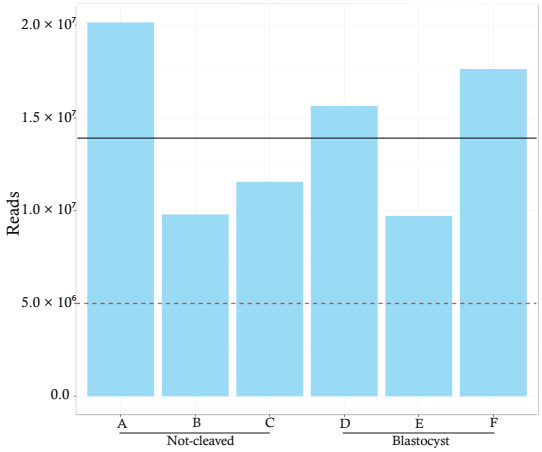


Figure 1: Total number of miRNA reads in each sample
Total number of miRNA sized reads for each sample of cumulus cells pooled from 10-16 bovine cumulus-oocyte complexes; on average 14 million reads were obtained per sample, as indicated by the black line. The red dotted line marks 5 million reads, the amount considered sufficient for meaningful analysis. A, B, C = cumulus pieces surrounding oocytes that failed to cleave after exposure to sperm; D, E, F = cumulus pieces surrounding oocytes that developed into a blastocyst after fertilization.

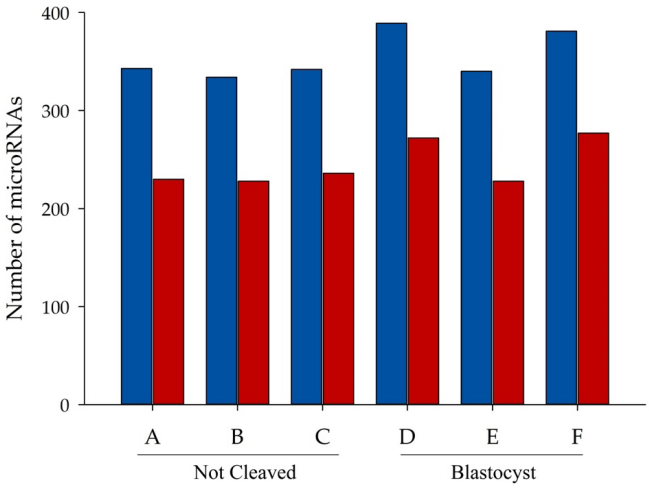


Figure 2: Number of miRNA reads
Number of known microRNAs with more than a given number of mapped reads: > 10 but < 50 reads per sample for blue bars and > 50 per sample for red bars. A, B, C = cumulus pieces surrounding oocytes that failed to cleave after exposure to sperm; D, E, F = cumulus pieces surrounding oocytes that developed into blastocysts after fertilization.

In short, cumulus cells harvested from the COCs of oocytes that failed to cleave after exposure to sperm, and those that yielded a blastocyst, showed a similar expression of miRNAs. Of all the identified miRNAs, miR-21-5p was the most abundant (> 2,000,000 reads, Table 2), but the number of reads was similar between the two groups. Different members of the let-7 family, miR-155 and miR-99a-5p also gave more than 100,000 reads in each group (Table 2).

miRNA	counts NC	counts Blast
bta-miR-21-5p	2,324,236	2,915,237
bta-let-7f	431,569	335,113
bta-let-7i	302,000	256,981
bta-let-7g	228,393	177,529
bta-miR-155	225,179	158,161
bta-let-7a-5p	217,033	158,238
bta-miR-99a-5p	129,482	112,348
bta-miR-30d	85,620	75,966
bta-miR-26a	73,820	56,991
bta-miR-320a	59,660	53,015
bta-miR-92a	55,108	47,255
bta-miR-10b	54,473	66,439
bta-miR-202	38,887	32,030
bta-miR-148a	38,574	40,607
bta-let-7b	36,653	29,117
bta-miR-532	34,632	32,815
bta-miR-99b	29,386	31,287
bta-let-7e	25,088	20,497
bta-miR-125a	22,159	20,436
bta-let-7c	23,668	17,542

Table 2: Most abundantly expressed miRNAs
List of the 20 most abundantly expressed miRNAs in bovine cumulus cells. NC – cumulus pieces enclosing oocytes that failed to cleave after exposure to sperm; Blast – cumulus pieces enclosing oocytes that developed into a blastocyst after fertilization.

When comparing the miRNAs expressed in cumulus cells from around an oocyte that developed into a blastocyst after fertilization with those surrounding an oocyte that had not cleaved, a high variability between the different pooled samples and no repeatable differences between the groups

were observed (Figure 3). Samples A and B (cumulus cells enclosing oocytes that did not cleave after exposure to sperm) and samples F and D (cumulus cells surrounding oocytes that developed into blastocysts) grouped together after hierarchical clustering. By contrast, samples C (cumulus cells enclosing oocytes that did not cleave) and E (cumulus cells surrounding oocytes that developed into blastocysts) exhibited a markedly different miRNA expression profile; this was also apparent after principal component analysis (Figure 4). In general, within the tested groups the expression of miRNAs was variable, which presumably contributed to the absence of differences in the expression profiles between the groups.

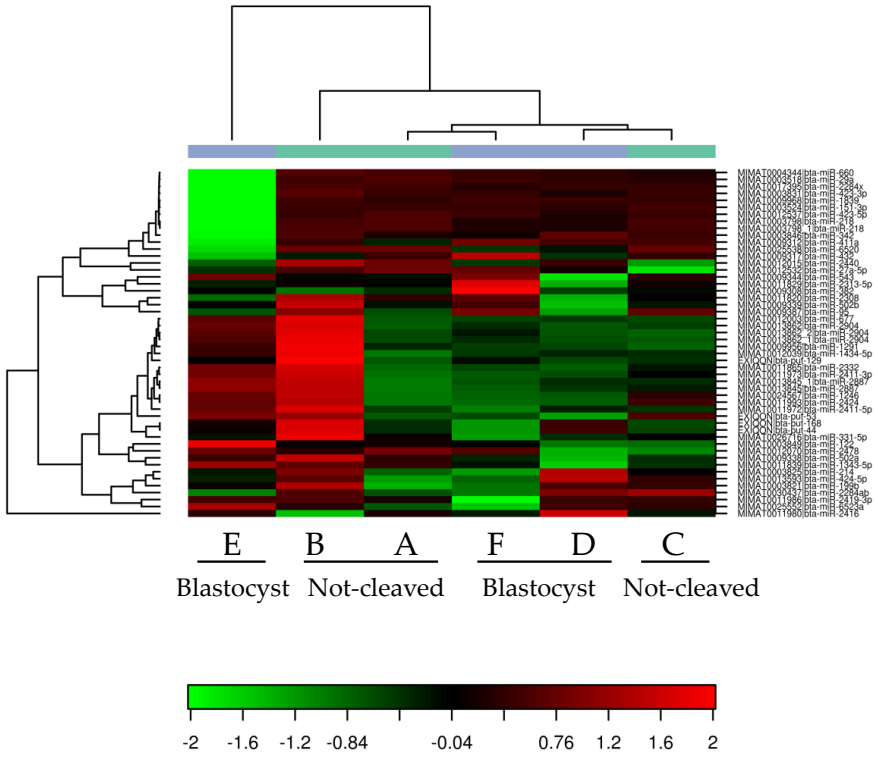


Figure 3: Heat Map of the tested cumulus cell samples
Heat Map and unsupervised hierarchical clustering by sample and microRNA. The clustering was performed on all samples, and on the top 50 microRNAs with the highest % CV based on TPM normalized reads. A, B, C – cumulus pieces surrounding oocytes that failed to cleave after exposure to sperm; D, E, F – cumulus pieces surrounding oocytes that developed into blastocysts after fertilization.

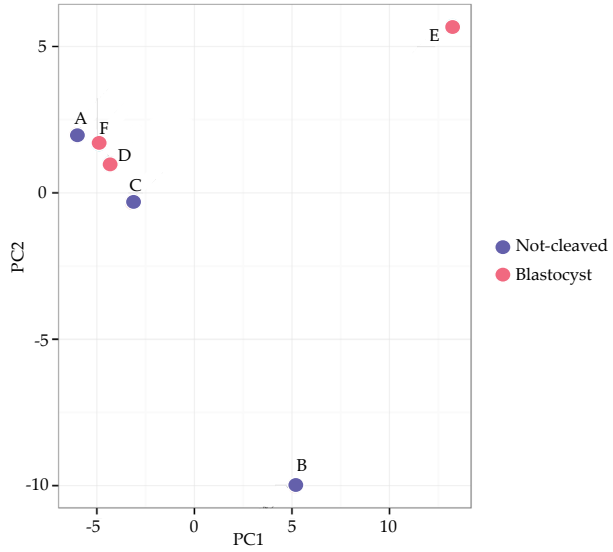


Figure 4: Principal component analysis (PCA) plot
The PCA was performed using the 50 miRNAs that had the largest variation across all samples. The features have been shifted to be zero centered, (i.e. the mean value across samples was shifted to 0) and scaled to have unit variance (i.e. variance across samples was scaled to 1) before the analysis. The groups do not cluster, not least because samples B and E differ greatly from the rest for the primary component, and from each other for the secondary component. Red circles = cumulus pieces surrounding oocytes that failed to cleave after exposure to sperm; blue circles = cumulus pieces surrounding oocytes that developed into blastocysts after fertilization.

	Not-cleaved			Blastocyst		
miRNA	**A**	**B**	**C**	**D**	**E**	**F**
bta-miR-214	35	33	30	119	22	13
bta-miR-218	4546	1451	2198	3004	86	1411
bta-miR-222	3228	1175	2090	1724	1291	1151
bta-miR-342	1267	802	963	2856	20	515
bta-miR-424-3p	354	62	166	159	208	181
bta-miR-424-5p	27	26	34	113	20	16
bta-miR-2284ab	32	20	41	70	12	13
bta-miR-2478	357	92	66	105	154	150
bta-let-7i	428545	199640	277815	3996881	225420	148643
bta-let-7g	332160	154890	198131	237141	163940	131506

Table 3: Sequencing counts of chosen miRNAs
Sequence counts for miRNAs examined further. Not cleaved = cumulus pieces surrounding oocytes that failed to cleave (samples A, B, C); Blastocyst = cumulus pieces surrounding oocytes that developed into blastocysts after fertilization (samples D, E, F).

qRT-PCR from pooled cumulus complex samples

Although miRNA expression did not differ significantly between the two groups, expression of miRNAs for which the abundance appeared to differ according to the heat map (Figure 3) was further examined by qRT-PCR. The selected miRNAs had low or moderate total reads at sequencing and, to compensate for the anticipated low expression levels, pooled samples of cumulus cells from individually cultured COCs were analysed. The expression of miR-214, miR-424 and 2284ab was below the level of detection by qRT-PCR and, indeed, the read numbers for these miRNAs were around or below 100 (Table 3). For miR-342, miR-2478 and miR424-3p, expression levels (Table 3) were similar in the cumulus cells surrounding an oocyte that gave rise to a blastocyst to those in cumulus cells around oocytes that did not cleave (Figure 5).

qRT-PCR from individual cumulus complex samples

For both the sequencing and the initial qRT-PCR analysis, pooled samples of cumulus cells from individually cultured COCs were used. However, since oocyte quality is likely to be dependent on many different factors acting at different times and on different pathways, pooling of the samples might obscure miRNAs that are involved in regulating oocyte competence. Therefore, expression levels of candidate miRNAs were determined using qRT-PCR in cumulus cells from individual COCs. The selection of miRNAs examined was based on the sequencing results; four of the selected miRNAs had been highly expressed and showed a moderate difference in expression between 'non cleaved' COCs and COCs that gave rise to a blastocyst, namely let-7g, let-7i, miR-222 and miR-218. For all four miRNAs, a high variability in miRNA expression between the twelve individual samples within a group was observed (Figure 6).

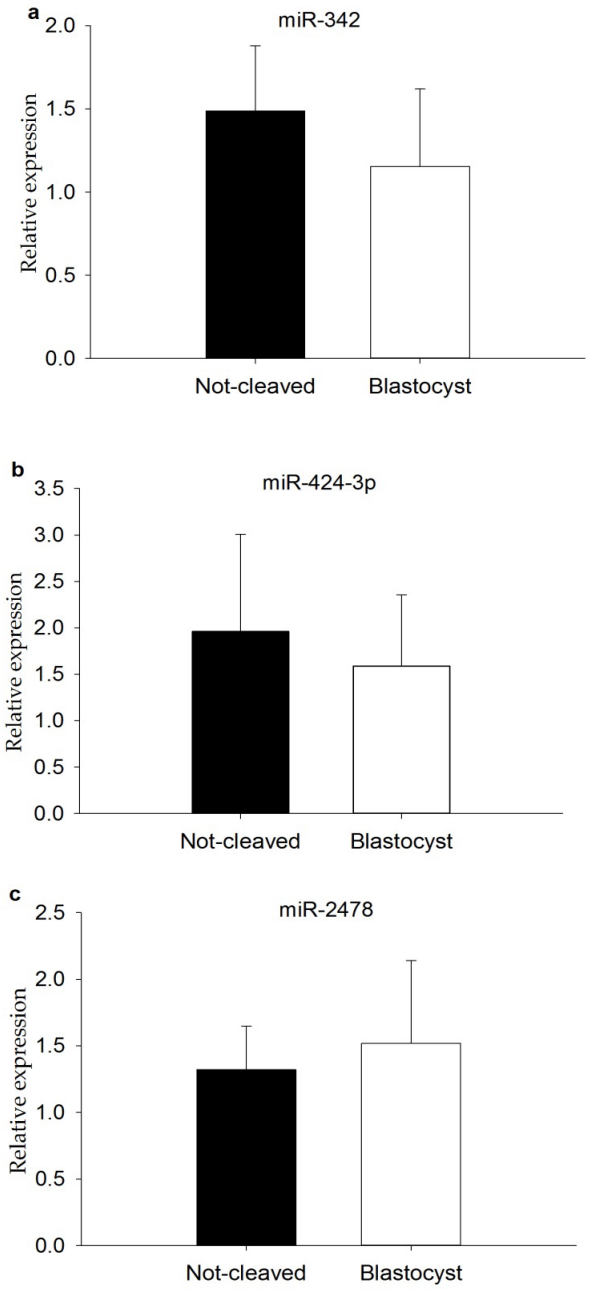
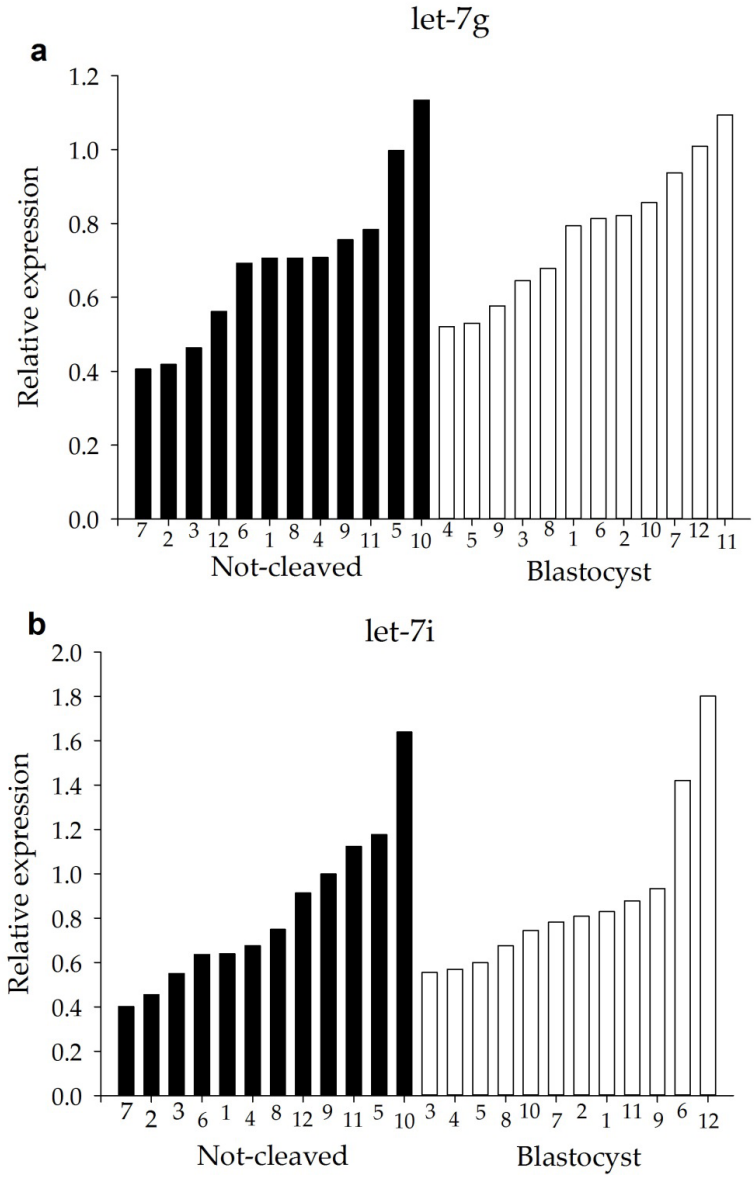


Figure 5: miRNA expression of pooled cumulus cell samples
Mean miRNA expression (qRT-PCR) for (A) miR-342, (B) miR-424-3p and (C) miR-2478 in pooled cumulus cell samples enclosing oocytes that failed to cleave (black bars) or that developed into blastocysts (white bars) after exposure to sperm. All samples were normalized for let-7a, miR-26a and miR-191 expression.

Finally, analysis of the sequencing data indicated 178 novel putative miRNAs. Not all of these miRNAs were detected in the cumulus cell samples; however 9 novel miRNA species were detected with a mean of >10 reads, indicating that the miRNAs are indeed generated and expressed (Table 4). However, the expression levels of these novel miRNAs did not differ between the non-cleaved and blastocyst groups.

Name	Chr	START	STOP	SEQUENCE
bta-put-45	chr7	12981779	12981801	CCGUGCCUACUGAGCUGAAACAC
bta-put-129	chr21	69641635	69641655	UGCAAGCAACACUCUGUGGCA
bta-put-53	chr7	53516556	53516579	UAUACUCUGAUUGGUUCAUUAUGA
bta-put-79	chr29	1067001	1067020	AUGGUCAUUACCAAGGCUUU
bta-put-168	chr7	5206807	5206828	UCAAAGUGAAUUUGGAGGUUCU
bta-put-44	chr7	5206802	5206823	UCAAAGUGAAUUUGGAGGUUCU
bta-put-82	chr29	41693966	41693989	GAUCCGCGUAAUGUACGGAGGUAG
bta-put-156	chr5	103546363	103546386	GGACCUCAGUUCCAAACCUCUGCC
bta-put-25	chr5	104238656	104238676	AUGUGGACCCAGGGAGCUGGG

Table 4: Novel expressed miRNAs
Predicted and expressed miRNAs identified from read data based on read count and secondary structure according to miRPara classification score. Description of chromosomal location is shown with Chr, Start, Stop.


a
let-7g
Relative expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
7 2 3 12 6 1 8 4 9 11 5 10 4 5 9 3 8 1 6 2 10 7 12 11
Not-cleaved
Blastocyst
b
let-7i
Relative expression
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
7 2 3 6 1 4 8 12 9 11 5 10 3 4 5 8 10 7 2 1 11 9 6 12
Not-cleaved
Blastocyst

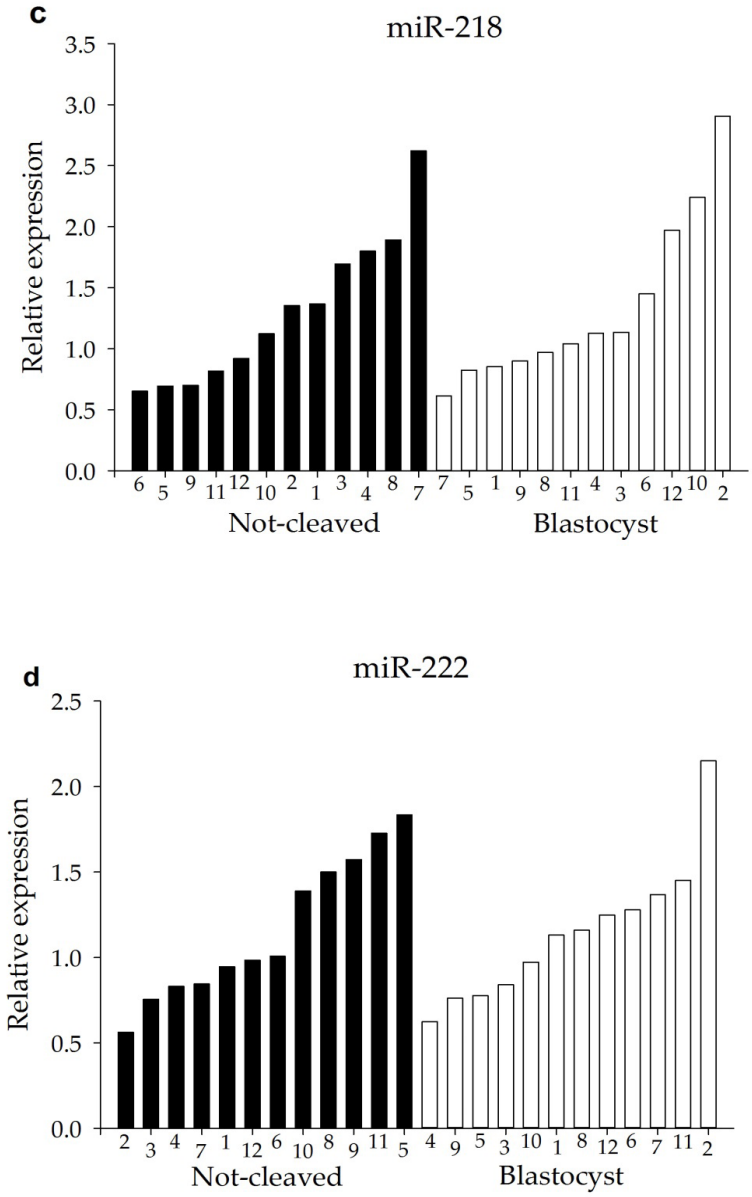


Figure 6: miRNA expression of individual miRNA samples
miRNA expression for cumulus cells from individual COCs. Expression levels of (A) let-7g, (B) let-7i, (C) miR-218 and (D) miR-222 as determined by qRT-PCR. X-axes depict individual cumulus complex samples from COCs that had enclosed an oocyte that either failed to cleave (black bars) or developed into a blastocyst (white bars) after exposure to sperm. All samples were normalized for expression of let-7a, miR-26a and miR-191.

Discussion

In this study, the miRNA expression profiles of cumulus cells from developmentally competent COCs were compared with those from around oocytes did not cleave after exposure to sperm. As far as we know, this is the first study to examine whether cumulus cell miRNA expression can be used to predict the quality of oocyte before fertilization. In our study, samples of cumulus cells from individually fertilized and cultured COCs were used, and retrospectively assigned to post-fertilization developmental competence groups.

For identification of miRNAs expressed in cumulus cells, next generation sequencing was used. From both groups, three pooled samples of cumulus cells, each consisting of cells from around 10 COCs, were analysed. Cumulus cells surrounding an oocyte that did not cleave after sperm exposure or developed into a blastocyst were chosen, to have the two most different stages to see a change in miRNA expression. However, no specific miRNA species were identified that could be correlated to oocyte quality, suggesting that miRNA expression in cumulus cells is not predictive of oocyte quality. Since our aim was to identify competent oocytes before fertilization, we decided to compare miRNA expression patterns in cumulus cells that did not cleave after exposure to sperm, versus those that developed into a blastocyst after exposure to sperm. This did not allow us to distinguish between those oocytes that were fertilizable, and those that were not. For the identification of cumulus markers that would predict embryo developmental competence, comparison between cells originating from embryos that remained at the two-cell stage versus those that become a blastocyst would be useful but this would always require fertilization and the generation of embryos. Oocyte fertilizability and developmental competence are dependent on various factors, and pooling of the cumulus pieces might have obscured any influence of individual miRNAs. In this respect, we further showed that the expression levels of several selected miRNAs varied greatly between cumulus complexes from individual COCs; at least for these miRNAs, the variation in expression was similar for competent and incompetent COCs. Oocytes from slaughterhouse ovaries were used in this study, which represent a heterogeneous population. Possibly, a more homogeneous oocyte population, for example originating from one breed, would reveal specific miRNAs. Our data indicate however that these could not be used as markers in a heterogeneous population, such as the human population.

A study of human oocytes reported a change in expression level between the germinal vesicle and the metaphase II stage for 15 of 722 miRNAs expressed during maturation [37], suggesting specific roles during maturation and early embryo development. It would be interesting to determine which miRNA expression levels change the most in bovine cumulus cell complexes during maturation. On a similar note, the difference in miRNA expression patterns between outer cumulus and inner cumulus, i.e. the cells furthest from or closest to the oocyte, might shed more light on the function of miRNA in cumulus cells.

Our sequencing results showed that miR-21-5p is the most abundant miRNA in bovine cumulus cells, followed by various members of the let-7 family and miR-155. In human cumulus cells, members of the let-7 family and miR-21 are also the most abundant miRNAs; however, their functions remain unclear [14]. An anti-apoptotic effect was proposed for miR-21 in mouse granulosa cells; however, it was not clear how this effect was achieved, because no change in apoptotic protein expression was observed after miR-21 knockdown [39]. A relatively low expression of miR-21 was detected in bovine oocytes, but the expression increased dramatically during early embryonic development [36]; similarly, a 25-fold increase in miR-21 expression was reported in pig cumulus cells during maturation [40]. A potential target of miR-21 is programmed cell death protein 4 (PDCD4), and interaction between miR-21 and PDCD4 has been reported in pig oocytes [40]. Whether miR-21 has a similar function in bovine cumulus cells remains to be determined.

Analysing miRNA expression in oocytes and cumulus cells using a PCR array showed a negative fold change of miR-155 expression between bovine oocytes and cumulus cells, indicating higher expression in cumulus cells than in oocytes. Interestingly, this fold change increased during maturation, indicating that either the expression levels in the cumulus cells decreased or the level in the oocyte increased, or both [33]. MiR-155 was also found to be abundantly expressed in exosomes recovered from bovine follicular fluid, and the expression level was higher in fluid from follicles that contained a growing oocyte compared to those with a fully-grown oocyte [41]. Based on the high expression levels of miR-155 in cumulus cells, it seems likely that this miRNA is produced by the cumulus cells themselves, although it cannot be excluded that cumulus cells take up miR-155 from the follicular fluid.

High expression levels of various let-7 family members have been reported in cumulus cells; among the most abundant let-7 family members were let-7f, let-7i and let-7g. Let-7 was also abundant in human and mouse cumulus cells [42]. Interestingly, let-7 miRNAs are negatively associated with pluripotency [43], although they are highly expressed in oocytes [35, 44]. This indicates a high degree of tissue specificity of miRNA expression and underlines the likelihood that they are involved in regulating different processes at specific time points.

Although target prediction methodologies are improving, computational prediction of miRNA targets needs to be experimentally validated since predicted target sequences can give rise to false positives. In this respect, examination of candidate gene expression after overexpressing or downregulation of miRNAs appears essential, preferentially combined with a demonstration of an interaction between the miRNA and its target mRNA, for instance using luciferase reporter constructs [45].

Overall, the results of this study suggest that the quality of an oocyte within a COC before fertilization cannot be predicted by the miRNA expression palette of the cumulus cells. However, the high and dynamically changing expression levels of various miRNA species suggest that they are important for cumulus cell function. Whether cumulus cell miRNAs are transported into the oocyte remains to be investigated. The identification of novel miRNAs expressed by cumulus cells emphasizes that much remains to be discovered about these regulatory molecules.

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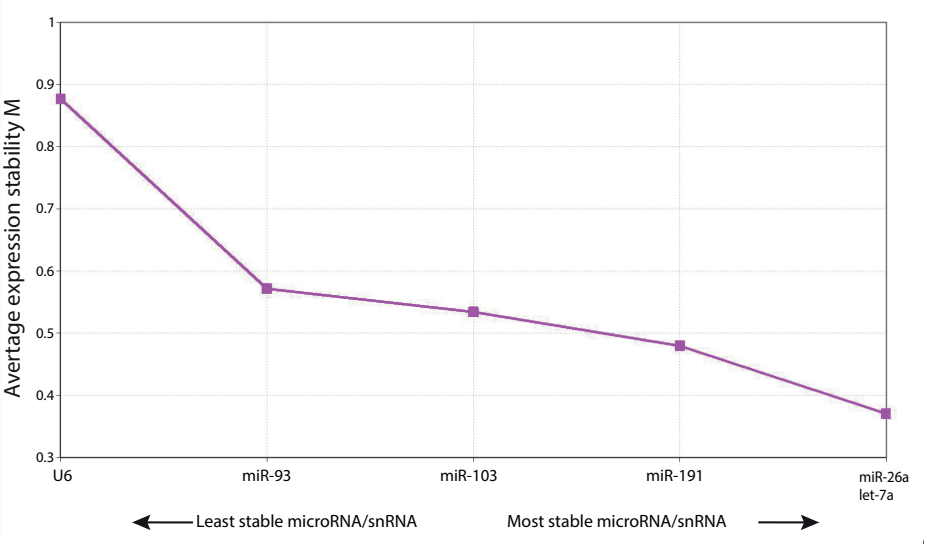
Supplementary Figure

Figure S1: Average expression stability of different mi/snRNAs
Average expression stability (M) of miRNAs/snRNA in bovine cumulus cells. The three miRNAs with the lowest M-values were selected for qRT-PCR normalization.




3

Bovine inner and outer cumulus cells show different patterns of miRNA and mRNA expression

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In preparation

Abstract

During oocyte maturation, the somatic cumulus cells that enclose the oocyte play an important role in the acquisition of developmental competence. How exactly the cumulus cells interact with the oocyte and influence maturation is however poorly understood. In particular, it is not known how cumulus cells assist the oocyte to develop the capacity to support post-fertilization development of an embryo and, ultimately, a neonate. We postulated that the pattern of cumulus cell miRNA and mRNA expression changes during the course of oocyte maturation, and differs between different populations of cumulus cells, namely the inner and the outer cells. The expression of miR-21, miR-25, miR-125 and miR-155 and of mRNA for *CD44*, *SMAD2*, *SLC38A2* and *WEE1* was determined by qRT-PCR. Expression of miR-21 and miR-155 changed over time, while miR-25 and miR-125 expression differed between inner and outer cumulus cells. The expression of the mRNAs examined changed over the course of maturation. Subsequently, a luciferase assay was performed to determine whether *CD44* or *SLC38A2* are targets of miR-25, and whether *SMAD2* or *WEE1* are targets of miR-155. However, no interaction between the selected mRNAs and miRNAs was found.

Introduction

An oocyte is considered developmentally competent when it has acquired the ability to develop into a blastocyst after fertilization, that in turn gives rise to a viable pregnancy culminating in live birth [1]. Critical steps in the oocyte's acquisition of developmental competence include the processes of nuclear and cytoplasmic maturation, which are initiated after meiotic resumption. The somatic cells that develop in a complex around the oocyte within the ovarian follicle, i.e. the cumulus cells, play a crucial role in supporting 'correct' oocyte maturation, particularly in the first 8h after meiotic resumption (Uhde *et al.*, submitted). The granulosa-derived cumulus cells form a multi-layer complex around the oocyte, in which the outer cells are at a much larger distance from the oocyte than the inner cells. The innermost cumulus cells (corona radiata) contact and communicate bidirectionally with the oocyte directly via transzonal cytoplasmic projections, which contain gap junctions and zona-adherens-like junctions [2–5]. These cytoplasmic projections are known to allow the transport of energy substrates, like cyclic adenosine monophosphate (cAMP) [6], and larger molecules including RNA, between the cumulus cells and the oocyte [3].

During maturation, the cumulus oocyte complex expands as a result of an increase in the hyaluronic acid rich matrix deposited by the cumulus cells [7]. If this process of cumulus expansion is impaired, uterine implantation of the resulting blastocysts is compromised [8]. Hyaluronic acid is synthesised by the enzyme hyaluronan synthase 2 (HAS2) [6,9]. In man, the expression of HAS2 in cumulus cells has been reported to correlate with embryo quality [10]. HAS2 expression is induced by oocyte secreted growth differentiation factor-9 (GDF-9), a TGFβ superfamily protein that activates SMAD2/3 signalling [11]. Studies in mice have shown that SMAD2 is important for proper cumulus complex expansion [12]. Another molecule essential for cumulus expansion is the cell surface adhesion molecule CD44, which is required for hyaluronic acid binding within the extracellular matrix [13]. CD44 and HAS2 in bovine cumulus cells have been proposed to play important roles in the acquisition of oocyte developmental competence [14].

Amino acids have also been shown to play an important role during oocyte maturation in cattle [15], pigs [16] and hamsters [17]. The transport of amino acids into cells occurs through specialized transporters, where each amino acid transporter has a specific range of substrates. The sodium-coupled

neutral amino acid transporter 2 (SLC38A2) transports neutral amino acids, including alanine and glutamine [18], and is expressed in both granulosa and cumulus cells, but not in the oocyte at the germinal vesicle (GV) stage.

Gene expression can be regulated in various ways, including post-transcriptional mRNA regulation (i.e. after production of the mRNA). Post-transcriptional mRNA regulation can be directed by microRNAs (miRNAs), a class of highly conserved non-coding RNAs with a length of 21-25 nucleotides. The sequence-specific binding of miRNAs to the 3'UTR of a target mRNA leads either to translational repression or to mRNA degradation [19]. While there is a degree of specificity, a single miRNA can regulate the expression of ~100 different mRNAs, and the mRNA(s) regulated is tissue-specific [20,21]. In this way, miRNAs can be involved in a multitude of cellular processes, including proliferation, differentiation and apoptosis [22] and they therefore play important roles in normal mammalian development [23]. During bovine [24] and human [25] oocyte maturation, miRNA expression changes. In addition, miRNA expression differs between oocytes and their surrounding cumulus cells. Since it is known that small RNAs can be trafficked via the transzonal processes, it has been suggested that miRNAs could play an important role in cumulus-oocyte communication [26].

Based on previous reports [27] of relatively high expression in cumulus cells, we investigated the expression of miRNA-21, miRNA-25, miRNA-125 and miRNA-155 in the inner cumulus cells by qRT-PCR. MiR-21 was by far the most expressed miRNA in cumulus cells, while the other tested miRNAs were stably expressed and among the top 30 expressed miRNAs in the previous study. The same technique was used to examine the expression of putative target mRNAs in the cumulus cells. Finally, several candidate target genes were tested for their regulation by the miRNAs miR-25 and miR-155 using a luciferase assay.

Material and Methods

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Collection of ovaries, COC recovery and in vitro maturation

Bovine ovaries were retrieved from a local slaughterhouse (Gosschalk, Epe, The Netherlands) and transported to the laboratory in a thermos flask. Cumulus-oocyte complexes were recovered and cultured as described previously [28]. In short, after arrival at the laboratory the ovaries were rinsed with tap water and subsequently maintained at 30°C in physiological saline (0.9%) containing 100 IU/ml penicillin and 100 µg/ml streptomycin. From small (2-8 mm) antral follicles, cumulus-oocyte complexes (COCs) were aspirated under low pressure using an 18-gauge needle connected via a 50 ml collection tube to an aspiration pump. COCs with several cumulus cell layers were selected and rinsed with HEPES buffered M199 (Gibco BRL, Paisley, UK). *In vitro* maturation was performed for groups of 50-60 COCs in 500 µl $NaHCO_3$-buffered M199 (Gibco BRL) supplemented with 1% Penicillin-Streptomycin (Gibco BRL), 0.05 IU Follicle-stimulating hormone/ml (Organon, Oss, The Netherlands) in 4-well culture plates (Nunc A/S, Roskilde, Denmark), for 23h at 39°C in a humidified atmosphere containing 5% CO_2.

Cumulus cell collection and RNA isolation

Outer cumulus cells were collected by repeatedly pipetting COCs while examining them continuously under a stereomicroscope. Oocytes with their remaining inner cumulus cells were then vortexed for 3 min, after which the oocytes were removed and the inner cumulus cells were collected into a tube. Cumulus cell samples were lysed using 350 µl lysis buffer (Exiqon A/S, Vedbaek, Denmark) containing 10 µl/ml -mercaptoethanol and stored at -80°C until further use.

The miCURY RNA isolation kit (Exiqon A/S) was used for total RNA extraction, following the user guidelines. In brief, samples were sheared with a 23-gauge needle and 200 µl 100% ethanol was added. The samples were loaded onto the provided columns and washed by various centrifugation steps. Bound RNA was eluted with 50 µl elution buffer.

First-strand synthesis and qRT-PCR miRNA

The universal cDNA synthesis kit II (Exiqon A/S) was used to perform first-strand synthesis, with 10 µl template RNA in a total volume of 20 µl, as described in the manufacturer's instructions. Samples were incubated for 1h at 42°C followed by 5 min at 95°C, followed by immediate cooling on ice.

PCR was performed in a total volume of 15 µl containing 1 µl cDNA, 1.5 µl locked nucleic acid (LNA) primer pair (Exiqon A/S), and 7.5 µl PCR master mix (BioRad, Hercules, CA, USA). Quantitative real time PCR was performed using a CFX Connect™ Real-Time PCR detection system (BioRad) using the following cycling conditions; 10 min at 95°C followed by 40 cycles each of 10 sec at 95°C and 60 sec at 60°C. Melting curves were plotted after each cycle series.

A standard curve for a 3-fold dilution series was obtained by plotting the log of the starting amount against the cycle threshold value for the dilution series. The geometric means of expression for the reference miRNAs miR-26a, miR-191 and let-7a were used for normalisation of target miRNA expression, as described previously [27].

First-strand synthesis and qRT-PCR mRNA

Isolated RNA from cumulus cells was treated with DNase (Qiagen, Venlo, The Netherlands) at a final concentration of 0.13 Kunitz U/µl. After 20 min at 37°C, the enzyme was inactivated by heating to 65°C for 10 min. For cDNA synthesis, Superscript III Reverse Transcriptase (Invitrogen, Groningen, The Netherlands) was used as per the manufacturer's instructions. QRT-PCR was performed in a total volume of 23 µl using a CFX Connect™ Real-Time PCR detection system (BioRad), with the following program: 3 min at 95°C, followed by 40 cycles of 10 sec at 95°C, 10 sec at the primer specific temperature (Table 1) and 20 sec at 72°C, and finally 1 min at 95°C. After each cycle series, melting curves were plotted. *PGK* and *GAPDH* [28] were used to normalize mRNA expression.

Vector cloning

Primers (Table 2) with predicted miRNA-specific binding sites in the 3'UTR of the target mRNA were used, including either a NheI or an XbaI restriction site. The amplified products were purified using a 1.8% agarose gel and a gel extraction kit (Qiagen, Hilden, Germany) as per the manufacturer's instruction. Purified products were ligated into the pmiRGlo vector (Promega, Madison, WI, USA) and transfected into competent NEB 5- Escherichia

coli (New England Biolabs, Ipswich, MA, USA). Recombinant plasmid DNA was analysed by restriction analysis and BigDye Terminator v1.1 Cycle Sequencing Kit (ABI, Frankfurt, Germany) and a pmiRGlo specific primer (5'-ACACGGTAAAACCATGAC-3').

Gene	Sequence	Annealing Temp.	Product size (bp)
CD44	F: GCCCTCCTGACGATGAGATG	61°C	111
	R: GTCTTGGTCTGGACGGGTG		
SMAD2	F: TGCTCTGAAATTTGGGGACTGA	63°C	103
	R: ACGACCATCTAGAGACCTGGTT		
WEE1	F: TGCTTCAAGGAAGAGTGCAGA	63.5°C	131
	R: GTGAAGAGTGCTCTTTCCTCAG		
SLC38A2	F: GCTCTGAAAAGCCATTATGCCG	63.5°C	116
	R: CCAAAGGAAGTAGTACCTGGATGAA		
HAS2	F: CAGACACCATGCTTGACCCA	64°C	348
	R: TCGTTGCATAGCCCAGACTC		
GAPDH	F: AGGCCATCACCATCTTCCAG	61°C	94
	R: GGCGTGGACAGTGGTCATAA		
PGK	F: CTGGACAAGCTGGATGTGAA	61°C	108
	R: AACAGCAGCCTTGATCCTCT		

Table 1: Primers used for qRT-PCR. F-Forward, R-Reverse

Gene	Sequence
CD44	F: GCTAGCGAGACGCGGAACTTGCAGAA
	R: TCTAGAGGCCCTCATTTCAGAAAGCAAT
CD44 non-specific	F: GCTAGCGGCCCTCATTTCAGAAAGCAAT
	R: TCTAGAGAGACGCGGAACTTGCAGAA
SMAD2	F: GCTAGCCGTTTACAGTGCTGTGGCAT
	R: TCTAGAGCCTGAGGTAAAGCTGGTCT
SMAD2 mismatch	F: GCTAGCGCCTGAGGTAAAGCTGGTCT
	R: TCTAGACGTTTACAGTGCTGTGGCAT
WEE1	F: GCTAGCGGTGGTGTGCTGCTTATGGT
	R: TCTAGACCAAAGGAGGGGAAGGAGGT
WEE1 mismatch	F: GCTAGCCCAAAGGAGGGGAAGGAGGT
	R: TCTAGAGGTGGTGTGCTGCTTATGGT
SLC38A2	F: GCTAGCTGTGGCTACACCAAATCAGG
	R: TCTAGAGCCTACAGCCATCAAATGCC
SLC38A2 mismatch	F: GCTAGCGCCTACAGCCATCAAATGCC
	R: TCTAGATGTGGCTACACCAAATCAGG

Table 2: Primers used for PCR to generate the inserts for the pmiRGlo vector

Luciferase assay

SAOS2 cells were plated onto 96-well plates in DMEM/F12 Glutamax (Gibco BRL) with 10% FBS (Gibco BRL) and 1% Penicillin-Streptomycin (Gibco BRL), incubated at 37°C for 24h and transfected using Effectene (Qiagen) as per the manufacturer's instructions. In short, a total volume of 100 µl was used for transfection, containing 0.25 µg DNA, 1 µl miRNA mimic (with a final concentration of 5, 10, 50 or 75 nM), 0.4 µl Enhancer and 1.25 µl Effectene reagent. Dual-Glo Luciferase Assay System (Promega) was used for cell lysis and luminescence measuring in accordance with the manufacturer's instructions. In brief, cells were lysed for 10 min by adding the Dual-Glo Reagent, followed by determination of firefly luminescence using a Berthold Centro LB 960 (Berthold Technologies, Vilvoorde, Belgium) plate reader for 10 sec. Subsequently, Dual-Glo Stop & Glo Reagent was added and after a 10 min incubation, renilla luminescence was measured using the same settings.

Statistical Analysis

Statistical analysis of blastocyst development was performed using SigmaPlot software (Systat software Inc., San Jose, CA, USA). Data are expressed as mean value ± SD. To compare the percentage of blastocysts between several treatment groups, analysis of variance (ANOVA) with a post hoc Bonferroni test was performed.

Results

Differential miRNA expression between inner and outer cumulus cells

Previously, we demonstrated that miRNA expression varies between cumulus cells harvested from individual cumulus oocyte complexes (COCs) [27]. Here, we examined the expression of several miRNAs in bovine cumulus cells during maturation, with the focus on differences in expression between inner and outer cumulus cells. The miRNAs miR-21, miR-25, miR-125 and miR-155 were selected because, in a previous study employing miRNA sequencing, we found these miRNAs to be relatively highly expressed in cumulus cells [27]. At the GV stage, miR-21 expression was similar between inner and outer cumulus cells. During maturation, miR-21 expression increased along a similar trajectory in inner and outer cumulus cells, significantly exceeding starting levels by the end of maturation (Figure 1a). MiR-25 expression

was higher in inner than outer cumulus cells at the GV stage and after 8h of maturation. By the metaphase II (MII) stage, miR-25 expression was similar between inner and outer cumulus cells (Figure 1b) largely because, while the expression of miR-25 was constant in outer cumulus cells over the course of maturation, it decreased in inner cumulus cells during IVM. The expression of miR-125 was stable during the whole maturation period but, at all stages examined, the expression was higher in inner than outer cumulus cells (Figure 1c). The relative amounts of miR-155 were very low in cumulus cells from GV stage oocytes; however, expression increased during the course of maturation, with no significant difference between the inner and outer cumulus cells (Figure 1d).

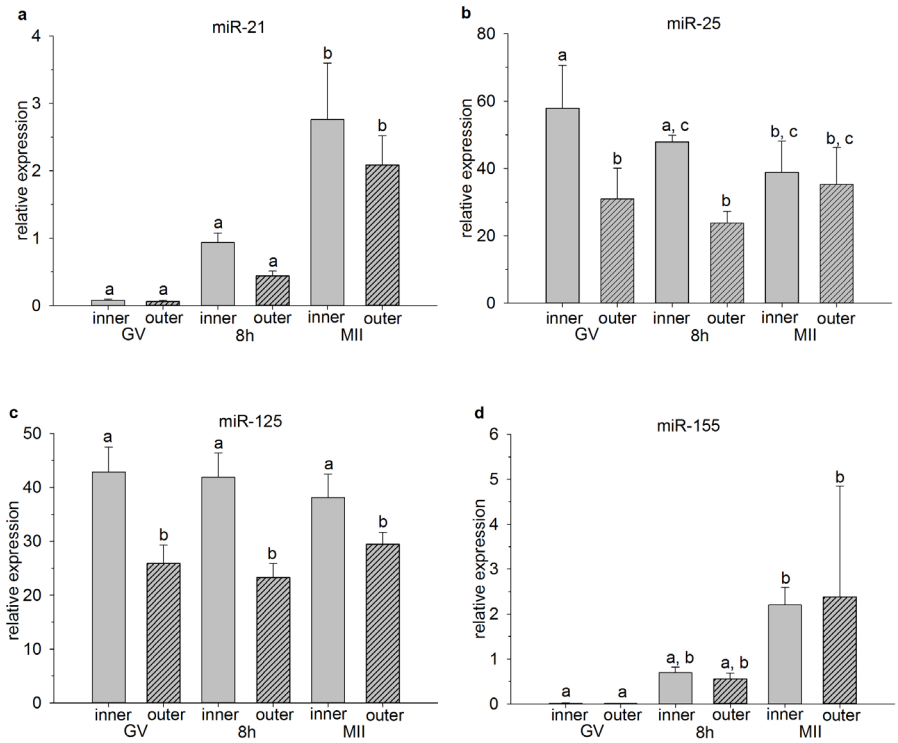

Figure 1: miRNA expression in cumulus cells
miRNA expression for bovine inner cumulus cells (inner) and outer cumulus cells (outer) at different time points during maturation. GV – germinal vesicle stage, 8h – 8h after onset of maturation, MII – MII stage. Expression of a) miR-21, b) miR-25, c) miR-125 and d) miR-155 were determined by qRT-PCR. Bars with different letters differ statistically ($P<0.05$).

Differential mRNA expression between inner and outer cumulus cells

Various studies have identified putative predictors of oocyte quality in cumulus cells, including HAS2 and CD44 [10,14,29]. By *in silico* analysis using Targetscan [30] and miRanda [31], we searched for candidate target mRNAs for the analysed miRNAs with possible relationships to oocyte quality. Genes that were predicted to be potential targets for the miRNAs examined included *HAS2*, *SMAD2*, *CD44*, *SLC38A2* and *WEE1*. *HAS2* expression was very low at the GV stage, both in inner and outer cumulus. During the first 8h of maturation, *HAS2* expression increased, particularly in the outer cumulus cells. By the MII stage, *HAS2* expression had decreased in the outer cumulus cells and was now similar to that in inner cumulus cells (Figure 2a).

SMAD2 expression was highest at the GV stage, in both inner and outer cumulus cells. During the initial phase of maturation, the expression of *SMAD2* decreased, however it plateaued between 8h of maturation and the MII stage. No differences in expression were observed between inner and outer cumulus cells (Figure 2b).

Low levels of *CD44* were expressed in GV stage cumulus cells, and expression increased significantly during the course of maturation with highest expression at the MII stage. At all time-points, *CD44* expression was similar between inner and outer cumulus cells (Figure 2c).

The expression of *SLC38A2* was similar between inner and outer cumulus cells at the GV stage and 8h after the start of maturation. Expression had increased by the MII stage, both in inner and outer cumulus cells but still did not differ between the two groups (Figure 2d).

WEE1 expression was reasonably constant throughout oocyte maturation and did not differ between inner and outer cumulus cells. A transient decrease in *WEE1* expression after 8h of maturation was only statistically significant for the outer cumulus cells (Figure 2e).

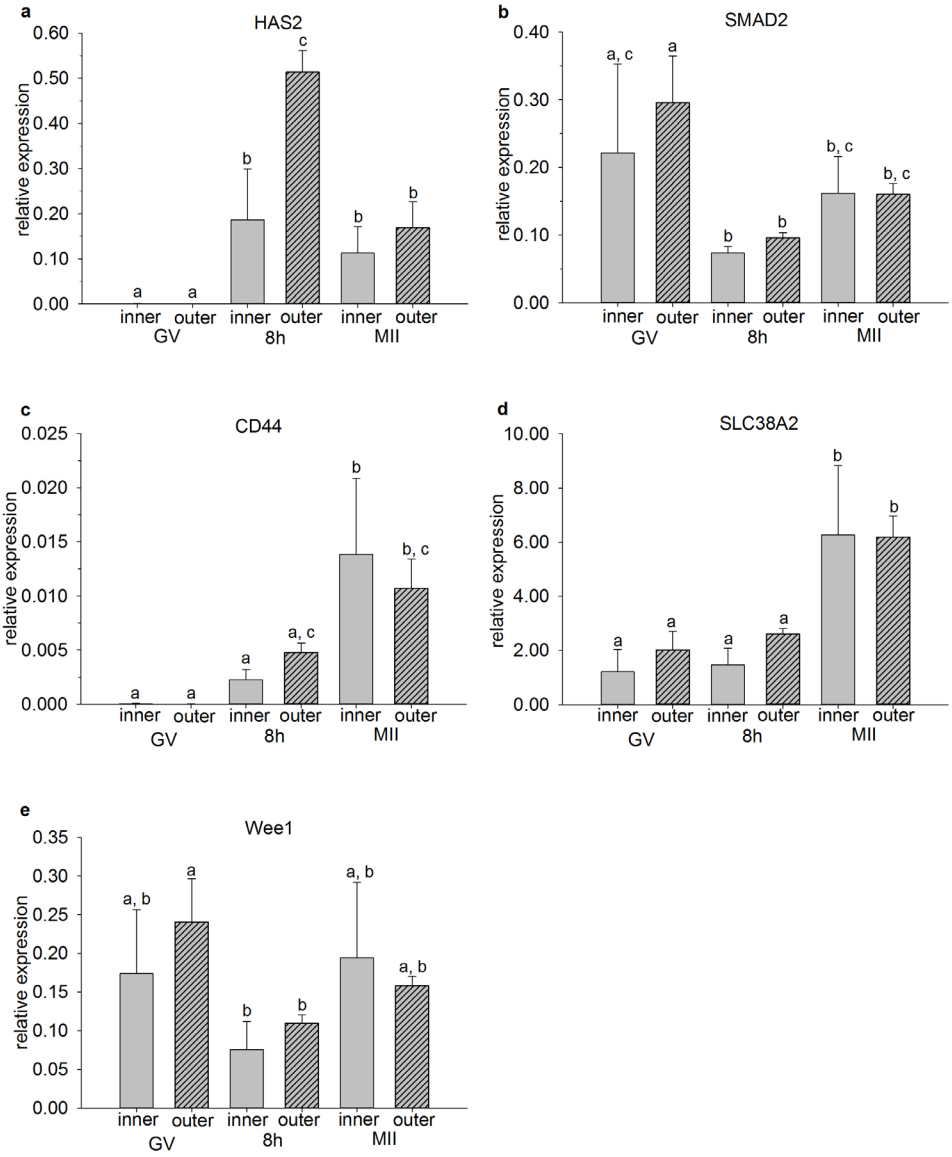

Figure 2: mRNA expression in cumulus cells
mRNA expression for bovine inner cumulus cells (inner) and outer cumulus cells (outer) at different time points during maturation. GV – germinal vesicle stage, 8h – 8h after onset of maturation, MII – MII stage. Expression of a) HAS2, b) SMAD2, c) CD44, d) SLC38A2 and e) Wee1 were determined my qRT-PCR. Bars with the same letters do not differ statistically (P>0.05).

Predicted target sequences are not regulated by the miRNAs

To verify whether *CD44* (Figure 3a) or *SLC38A2* (Figure 3b) were targets for miR-25, a reporter gene assay was conducted in cells form the commercial human osteosarcoma cell line, SAOS2. Expression of *CD44* and *SLC38A2* was not altered by miR-25 mimic administration. The same experimental approach was used to examine whether *SMAD2* and *WEE1* were targeted by miR-155. Administration of miR-155 mimics did not alter *SMAD2* (Figure 3c) or *WEE1* (Figure 3d) expression.

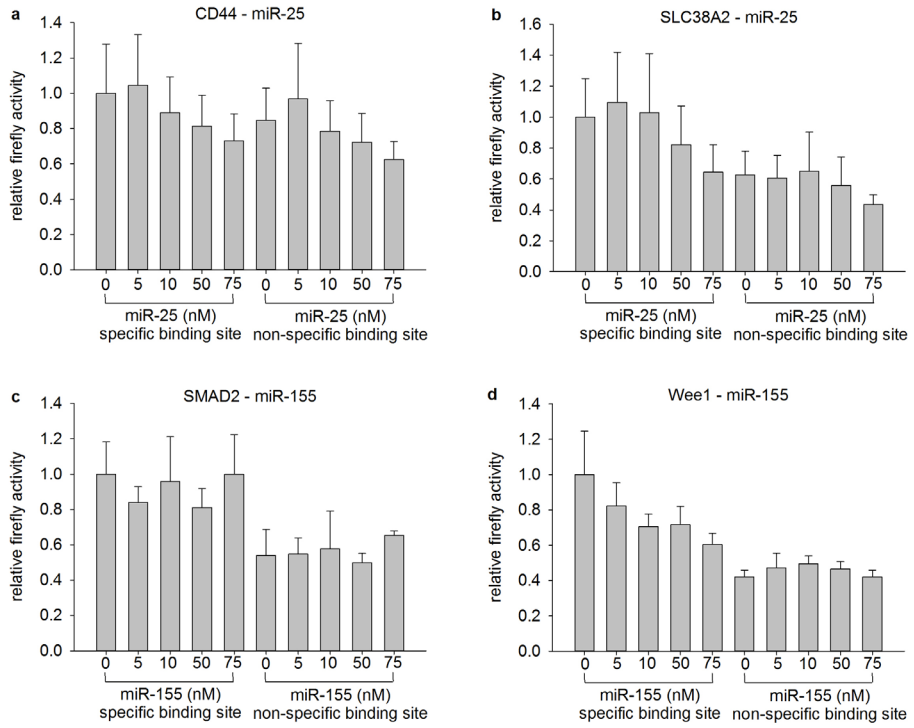

Figure 3: Luciferase assays

Luciferase assays for CD44 and SLC38A2 as possible targets of miR-25, and SMAD2 and Wee1 as possible targets of miR-155. a) Relative firefly activity of CD44 with different concentrations of miR-25 (0 – 75 nM); b) relative firefly activity of SLC38A2 with different concentrations of miR-25 (0 – 75 nM); c) relative firefly activity of SMAD2 with different concentrations of miR-155 (0 – 75 nM); d) relative firefly activity of Wee1 with different concentrations of miR-155 (0 – 75 nM).

Discussion

Based on previous miRNA sequencing results from cumulus cells [27], four miRNAs were selected for further examination of differences in expression between inner and outer cumulus cells. The most highly expressed miRNA (number of reads) in bovine cumulus cells was miR-21. MiR-155 was the fifth most abundant miRNA, after several let-7 family members [27]. MiR-25 and miR-125 were stably expressed and among the top 30 most abundant miRNAs in bovine cumulus cells. Although expression of these miRNAs was not correlated with developmental competence of the enclosed oocyte [27], it is possible that miRNAs from the innermost cumulus cells target oocyte mRNAs, whereas miRNAs from the outer cumulus cells are unlikely to reach the oocyte. Therefore, the expression of miRNAs was investigated over the course of maturation and compared between inner and outer cumulus cells.
During maturation, the oocyte secretes cumulus-expansion enabling factors (CEEFs) which, together with gonadotropins and epidermal growth factor-like peptides, initiate the expansion of the cumulus complex [6]. The concentration of CEEFs will be higher closest to the oocytes, and they will encounter the inner cumulus cells first. It is therefore likely that cell function, mRNA and miRNA expression patterns differ between inner and outer cumulus cells. In human cumulus cells, the expression of various miRNAs differs between the inner corona radiata cells and the outer cumulus cells [32]. Similarly, a gradient of anti-apoptotic factors was identified in cumulus cells [6]. Here, we identified differences in expression of miR-25 and miR-125 between inner and outer cumulus cells. Previously, downregulation of miR-25 has been reported to reduce proliferation of cancer cells [33]. MiR-125 is known to regulate 'maternal effect genes' in oocytes and early embryos involved in embryonic genome activation, like Sebox and Lin-28a [34]. Maternal effect genes are genes producing RNAs or proteins in the oocyte or zygote before zygotic gene activation starts. Since miR-125 was detected at higher levels in inner cumulus cells, we speculate that it is transferred to the oocyte to regulate maternal effect genes.

A major component of the cumulus cell matrix is hyaluronic acid, synthesis of which is encoded by the *HAS2* gene. The dynamics of *HAS2* expression in inner and outer cumulus cells are more or less correlated with the stage of maturation. At the start of meiotic resumption, the cumulus complex is compact, it then expands as meiosis progresses. The increase in *HAS2* expression during the first 8h of maturation may initiate cumulus expansion.

HAS2 expression was highest in the outer cumulus cells that play a greater role in expansion than the inner cells. At the MII stage, the cumulus was already fully expanded, which would make *HAS2* expression less important. A study on bovine cumulus cells showed an expression gradient of the anti-apoptotic factors, bone morphogenetic protein (BMP)-6 and -15 from the inner corona cells to the outer cumulus cells. This gradient inversely correlated to apoptosis of cumulus cells, which was more frequent among the outer cells than the inner cumulus cells [35]. Hyaluronic acid is one of the main ligands of CD44, a cell adhesion receptor involved primarily in cell adhesion and inhibition of apoptosis [36]. *CD44* was also detected in human cumulus cells, with a higher expression in cells of matured cumulus complexes compared to immature complexes [37], similar to our *CD44* expression results. Like HAS2, cumulus cell CD44 is thought to be important for the developmental competence of the enclosed oocyte [14]. BMP-6, BMP-15 and GDF-9 all belong to the TGFβ superfamily and are secreted by the oocyte. GDF-9 leads to SMAD2/3 activation, which is important for cumulus expansion [11,12,38].

In summary, even though cumulus cells can appear to be a homogeneous cell mass surrounding the oocyte, our results show that gene and small RNA expression differ between the inner and outer cumulus cells. This indicates that the cumulus cells are a heterogeneous population which probably have different functions and express different regulatory factors depending on their exact location within the cumulus complex and proximity to the oocyte.

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4

Metabolomic profiles of bovine cumulus cells and cumulus-oocyte-complex-conditioned medium during maturation *in vitro*

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Abstract

Cumulus cells are essential for nutrition of oocytes during maturation. In the absence of cumulus cells during maturation, oocyte developmental competence is severely compromised. In this study, we matured bovine cumulus-oocyte-complexes (COCs) for 8h, the cumulus cells were removed and denuded oocytes were further matured for 15h in either the medium conditioned by the initial 8h culture, or in fresh unconditioned medium. Denuded oocytes that completed maturation in conditioned medium demonstrated superior developmental potential to denuded oocytes that completed maturation in fresh medium. An inventory was made of the metabolites secreted by COCs into the maturation medium during the first 8h, from 8 to 23h, and during an entire 23h maturation protocol; the metabolomic changes in the cumulus cells during maturation were also investigated. In maturation medium, 173 biochemical components were detected compared to 369 different metabolites in cumulus cells. Significant changes in metabolomic components were evident in maturation medium and in cumulus cells during maturation, with most of the changes related to amino acid, carbohydrate, and lipid metabolism. The importance of two detected biochemicals, creatine and carnitine, for oocyte maturation was further investigated. The presence of carnitine, but not creatine during oocyte maturation improved oocyte developmental competence.

Introduction

The mammalian oocyte is surrounded by specialized somatic cells, cumulus cells, that support oocyte maturation and allow it to be fertilized and develop into a viable embryo. The final stage of oocyte maturation includes resumption of meiosis and storage of mRNA, proteins and nutrients required to support the early stages of embryo development before the embryonic genome is switched on. Cumulus cells are connected to the oocyte via transzonal cytoplasmic projections which enable the transfer of molecules between cumulus cells and the oocyte [1–5]. Maturation of oocytes in the absence of cumulus cells dramatically reduces the likelihood of fertilization and the competence to form an embryo, demonstrating the importance of the cumulus cells [6]. Nevertheless, during the first 3 hours of maturation there is already partial loss of transzonal cumulus cells processes [7–9], indicating that communication through the cytoplasmic connections across the zona pellucida is particularly important during the early stages of oocyte maturation. Furthermore, culture of denuded oocytes in the presence of cumulus-oocyte-complexes (COCs) partially restores the developmental competence of oocytes [10] indicating that, as well as gap junctional communication, factors secreted by the cumulus cells also positively influence oocyte development.

The exact components produced by cumulus cells that enhance the developmental competence of oocytes during maturation are, however, largely unknown. A better understanding of the functions of cumulus cells and cumulus cell products in the maturation of the enclosed oocyte may help to improve the success of *in vitro* maturation and fertilization.

To investigate the paracrine interactions between somatic cells and the oocyte, the developmental competence of bovine oocytes denuded 8h after the onset of *in vitro* maturation (IVM) and thereafter matured in either fresh or COC-conditioned culture medium was examined. Oocytes were denuded 8h after the start of maturation, because of the timing of the GVBD, which occurs between 6.6 and 8h [11]. Additionally, the communication between the cumulus cells and the oocyte is completely lost after 9h of maturation [12].

In addition, the metabolic profiles of cumulus cells and COC-conditioned media were determined by mass spectrometry to identify candidate components or changes that may support the oocyte's acquisition of developmental competence.

Materials and Methods

All chemicals were obtained from Sigma-Aldrich (St. Louis, Missouri, USA), unless otherwise stated.

Collection of ovaries, harvesting COCs, *in vitro* maturation and fertilization

Bovine ovaries were obtained from a local slaughterhouse and transported in a thermos flask to the laboratory. On arrival at the laboratory, the ovaries were rinsed in tap water and held at 30°C in physiological saline (0.9%) supplemented with 100 IU penicillin and 100 µg streptomycin per ml. COCs were aspirated from small antral follicles (2-8 mm) using an 18-gauge needle connected to a low pressure vacuum pump via a 50 ml collection tube, as described by van Tol *et. al.*[13]. Only oocytes with multiple layers of cumulus cells were selected for *in vitro* maturation, and rinsed with HEPES buffered M199 (Gibco BRL, Paisley, UK). The obtained COCs were randomly distributed into groups of 50-60 in 4-well culture plates (Nunc A/S, Roskilde, Denmark) containing 500 µl $NaHCO_3$-buffered M199 (Gibco BRL) supplemented with 1% (v/v) penicillin-streptomycin (Gibco BRL), 0.02 IU FSH/ml (Sioux Biochemical Inc., Sioux Centre, IA, USA), 0.02 IU LH/ml (Sioux Biochemical Inc.), 7.7 µg/ml cysteamine, and 10 ng/ml epidermal growth factor. Maturation involved culture for 23h at 38.5°C in a humidified atmosphere containing 5% CO_2.

For *in vitro* fertilization, frozen thawed sperm were selected by centrifugation through a discontinuous percoll (90/45%) gradient. Spermatozoa were added at a final concentration of 1×10^6 cells/ml to fertilization medium [14] supplemented with 1.8 IU/ml heparin, 20 µM d-penicillamine, 10 µM hypotaurine, and 1 µM epinephrine. Fertilization incubation was performed for 18-22h at 38.5°C in a humidified atmosphere containing 5% CO_2.

Presumptive zygotes were vortexed for 1 min to remove adhered sperm, transferred to synthetic oviductal fluid (SOF) [13] and cultured at 39°C in a humidified atmosphere, containing 5% CO_2 and 7% O_2. On day 5 of culture, cleaved embryos only were transferred to a new well containing fresh SOF, and cultured further until day 8.

Creatine and carnitine supplementation

To examine the effects of carnitine and creatine during oocyte maturation, various concentrations of either were added to the maturation medium. The final concentrations used were 2.5 mM, 5 mM, and 10 mM L-Carnitine-hydrochloride and 5 mM, 10 mM, and 20 mM Creatine-monophosphate. Two different groups of oocytes were exposed to these concentrations of carnitine or creatine; first, oocytes matured as intact COCs for 8h, that were vortexed for 3min to remove the cumulus cell, before being returned to fresh maturation medium supplemented with creatine or carnitine or, second, oocytes denuded before the start of maturation and subsequently matured for 23h with creatine or carnitine in the medium.

Control groups included intact COCs and denuded oocytes matured for 23h, and oocytes matured with an intact cumulus for 8h, and then denuded by vortexing for 3min before being returned to their original conditioned medium or transferred to fresh maturation medium. Each experiment was performed three times independently.

Collection of cumulus cell and maturation media

Cumulus cells were collected immediately after COC isolation for germinal vesicle stage oocytes, 8h and 23h after the start of *in vitro* maturation. COCs were denuded by recurrent pipetting through a narrow pipette, followed by vortexing and centrifugation.

Conditioned media were collected after 8h and 23h of COC culture. Media and cumulus cells were snap frozen in liquid nitrogen and stored at -80°C until further analysis. Cells and media were from four biologically independent experiments.

Viability assay

To assess the viability of cumulus cells 15 COCs were cultured in 100 µl maturation medium in a 96-well plate (Nunc A/S) and incubated at 38.5°C. To determine the activity, 10 µl AlamarBlue solution (Thermo Fisher, Waltham, MA, USA) was added either directly or after 21h of maturation and cells were cultured for an additional 2h. Fluorescence was subsequently measured with a DTX 880 Multimode Detector (Beckman Coulter, Woerden, The Netherlands) with an excitation wavelength of 535 nm and an emission wavelength of 590 nm. The sample signals were background corrected and viability determined after 23h as percentage of the fluorescence values

determined at the beginning of maturation. Three individual assays with ten COC replicates each were performed for each time point.

Metabolic analysis

Sample preparation, ultrahigh performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) and bioinformatics analysis were performed by Metabolon Inc. (Durham, NC USA) [15]. For missing values, minimum value imputation was used to calculate the fold-changes.

Briefly, the MicroLab STAR® system from Hamilton Company was used to prepare samples for four different analytical methods. The samples were analysed by two separate reverse phase (RP/UPLC)-MS/MS methods using positive ion mode electrospray ionization, one involving RP/UPLC-MS/MS with negative ion mode electrospray ionization, and one involving hydrophilic interaction chromatography (HILIC)/UPLC-MS/MS with negative ion mode electrospray ionization.

Raw data was extracted, peak-identified, and QC processed using Metabolon's hardware and software.

Statistical analysis

Statistical analysis of cleavage and blastocyst formation was performed using SPSS version 24 (SPSS Inc., Chicago, Il, USA). A logistic regression for grouped data was used to analyse success of cleavage and blastocyst formation, with condition and experimental run included as fixed factors. Data are presented as mean ± SD. A P value <0.05 was considered to be statistically significant.

Welch's two-sample t-test was used two compare metabolite concentrations in the cumulus cells and medium at different time points.

Data availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Results

COCs secrete maturation enhancing factors during the first 8h of maturation

To examine the influence of COC secretions on the acquisition of oocyte developmental competence during maturation, bovine oocytes were isolated from the ovaries of slaughtered cows and matured *in vitro* in four different groups:1) 23h as intact COCs; 2) denudation followed by 23h IVM in the absence of cumulus cells; 3) 8h IVM as COCs, followed by denudation and return to their own conditioned medium or 4) transfer to fresh medium for the remaining 15h of IVM (Figure 1a). After maturation, oocytes were fertilized *in vitro*, and the percentage that cleaved and developed to the blastocyst stage were determined on day 5 and 8 of culture, respectively.

The proportion of oocytes matured as COCs for 23h that cleaved was 78%, whereas oocytes matured without cumulus were clearly compromised since cleavage was observed in only 50% ($p<0.001$). Also, oocytes matured for 8h, denuded and placed into fresh maturation medium showed a compromised cleavage of 64%. However, when oocytes were matured within the COC for 8h, denuded and placed back into conditioned medium, the proportion undergoing cleavage was similar (84%) to that of oocytes matured as a COC for the entire 23h (Figure 1b).

The critical role of cumulus cells during oocyte maturation was confirmed by the difference in the proportion of oocytes matured within the COC that developed into blastocysts compared to oocytes matured without cumulus (50% and 3% respectively; $p<0.001$). When oocytes were denuded after 8h of maturation and placed back into COC-conditioned medium, the incidence of blastocyst formation was improved compared to oocytes transferred to fresh medium (26% and 2% respectively; Figure 1c). Whether conditioned medium from other cells, for example fibroblasts, oviductal cells or Buffalo Rat Liver cells can support preimplantation embryo development or would be beneficial for oocyte quality remains to be established. These data indicate that, in addition to direct contact between the oocyte and cumulus cells, factors secreted by COCs during the first 8h of maturation are important for the acquisition of oocyte developmental competence during the remaining 15h of maturation.

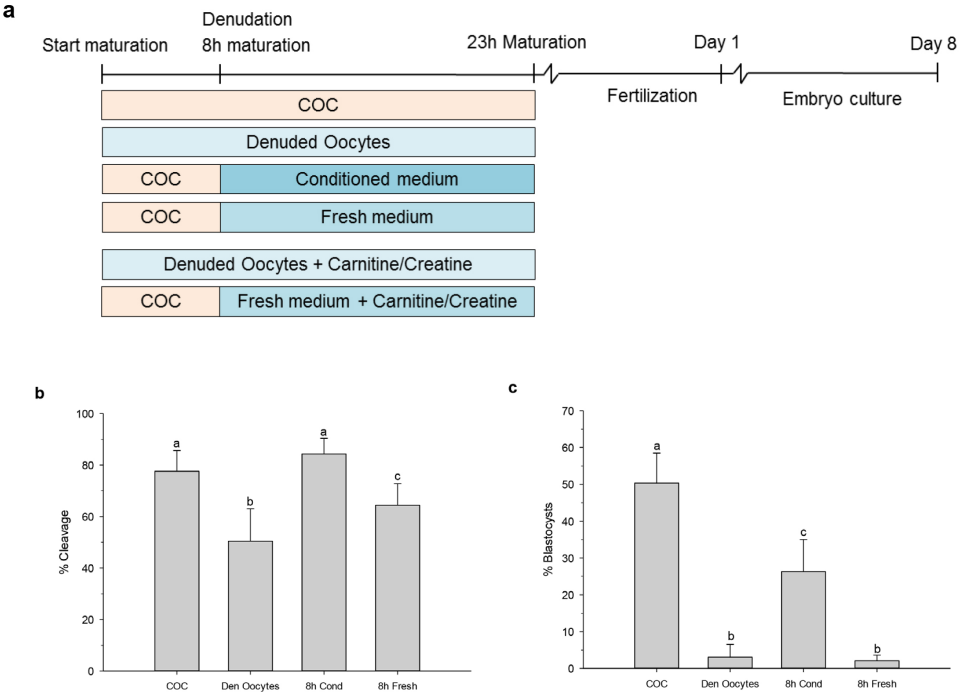

Figure 1: Experimental timeline (a) and developmental assessment after fertilization (b, c). a) Intact COCs or denuded oocytes were matured for 23h. Groups of intact COCs were denuded 8h after the start of maturation and either placed back into their original medium or placed into fresh maturation medium. In addition, maturation media were supplemented with creatine or carnitine as indicated. After 23h of maturation oocytes were fertilized and 5 days later cleavage was assessed; at day 8 after fertilization, blastocyst formation was assessed. Cleavage percentages of oocytes (b) and blastocyst formation of cleaved oocytes (c) matured differentially; as intact cumulus oocyte complexes (COC) for 23h; as denuded oocytes (Den Oocytes) for 23h; or matured as intact COCs for 8h, denuded and placed back in their own conditioned medium (8h Cond) or placed into fresh maturation medium (8h Fresh). Data are presented as mean ± SD. Different letters above bars indicate outcomes that differ significantly (N=6; $P<0.05$).

Identification of factors secreted by COCs during oocyte maturation

To identify factors in cumulus cells and COC-conditioned medium that are important for development of oocyte developmental competence, cumulus cells were collected before the start of maturation (oocyte germinal vesicle stage: GV), and at 8h and 23h after the start of maturation and subjected to metabolic profiling using ultrahigh performance liquid chromatography-

tandem mass spectroscopy. In addition, medium samples conditioned by COCs were collected after 8h and 23h. The metabolic profiles were compared to that of control (unconditioned) maturation medium.

To ensure that the cells remained metabolically active during maturation, a cell viability was determined before and 23h after *in vitro* maturation. The viability of the cumulus cells at 23hrs of maturation was 112% (±19 SD) from that of cells at the beginning of maturation indicating that the cells remained viable throughout maturation.

Unsupervised principal component analyses (PCA) of the metabolic profiles of cumulus cells showed that the different groups cluster separately (Figure 2a), indicating that the metabolites in cumulus cells changed over time in IVM. Similarly, PCA of conditioned medium yielded three clearly separated clusters for the different time points (Figure 2b) indicating that the nature of metabolites secreted by COCs into maturation medium also changed over time.

In cumulus cells, 369 different biochemical components were detected. During the first 8h of maturation, the levels of 41 of these components increased (Figure 3a) and the levels of 61 decreased (Figure 3b). Of these 41 and 61 components, 23 and three, respectively, changed exclusively during the first 8h of maturation (Figure 3a, b).

In COC-conditioned maturation medium, 173 different biochemical components were detected. Over the first 8h of maturation, the levels of 60 biochemical components increased (Figure 3c) whereas the levels of 13 decreased (Figure 3d). The concentrations in conditioned medium of ten components increased exclusively during the first 8h of maturation, whereas the levels of three factors decreased over the same time interval (Figure 3c, d).

Amino Acid Metabolism

Supplementation of amino acids to defined maturation medium has previously been shown to support the acquisition of development competence by the oocyte [16]. During maturation, the concentrations of nearly all detectable amino acids increased in the cumulus cells (Table S1; Figure S1); however, only a small number, including glutamine, leucine, and isoleucine increased significantly during the first 8h of maturation (Table 1). Interestingly, while the concentrations of glutamine and leucine increased during the first 8h of maturation, no further increase was observed during the remaining 15h

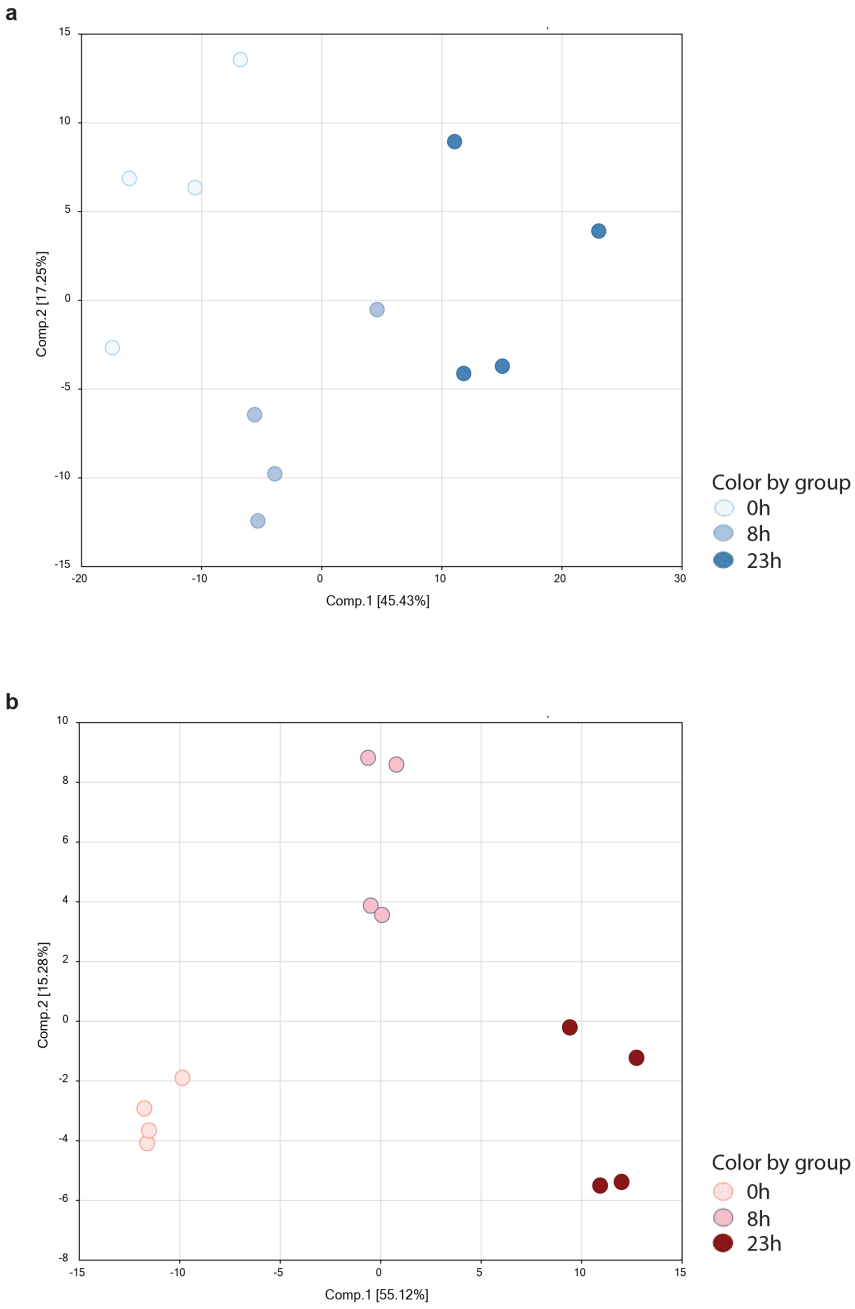

Figure 2: Principal component analyses of cumulus cells and maturation medium
Principal component analyses for a) bovine cumulus cells at GV stage (white), 8h (light blue) and 23h (blue) after start of maturation and b) control maturation medium (0h, light pink) and 8h (dark pink) or 23h (bordeaux) conditioning with maturing COCs.

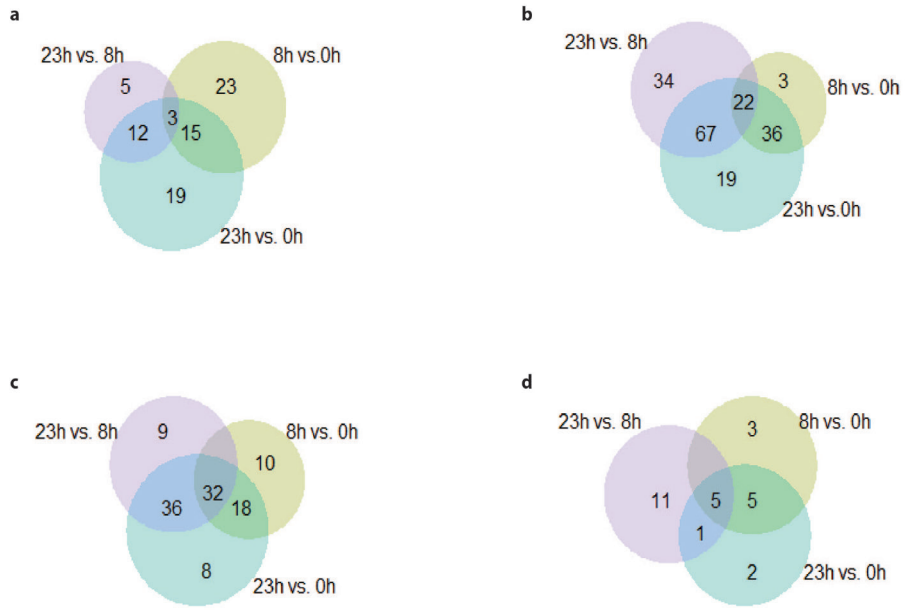

Figure 3: Venn diagram of significantly changed metabolites
Venn diagram showing numbers of metabolites with significantly (Welch's Two-Sample t-Test) altered concentrations in cumulus cells (a, b) and COC-conditioned medium (c, d) at different time points (8h vs 0h; 23h vs 0h and 23h vs 8h). a) Increased and b) decreased concentrations in cumulus cells. c) Increased and d) decreased concentrations in conditioned maturation medium.

of maturation. The levels of all detectable amino acids had increased by 23h of maturation (Table S1), with the exception of serine, the levels of which decreased between 8h and 23h of maturation. During the first 8h of maturation, the levels of betaine, N-trimethyl 5-aminovalerate, taurine, and ophthalmate in cumulus cells decreased dramatically (Table 1) and their concentrations remained reduced throughout the remainder of the 23h maturation (Table S1).

Changes in maturation medium conditioned by COCs for 8h or 23h were dominated by increases in the concentrations of various amino acids, including hypotaurine and ornithine (Table 2). The concentrations of creatine, a substrate for amino acid production, increased 427-fold in maturation medium after 8h (compared to 0h) and remained increased (516-fold) until the end of IVM (23h versus 0h; Table S2). Creatine is derived from

Table 1

Cumulus cells			Fold change	
Pathway	**Sub Pathway**	**Biochemical Name**	**8h vs. 0h**	**23h vs. 8h**
Amino Acid	Glycine, Serine, and Threonine Metabolism	betaine	0.06	1.40
		serine	1.18	0.58
	Glutamate Metabolism	glutamine	3.03	1.00
		pyroglutamine	0.37	4.62
	Lysine Metabolism	5-(galactosylhydroxy)-L-lysine	1.17	0.55
		2-aminoadipate	2.21	1.26
		N-trimethyl 5-aminovalerate	0.08	0.53
	Leucine, Isoleucine, and Valine Metabolism	leucine	1.78	1.73
		isoleucine	2.48	2.17
	Methionine, Cysteine, SAM, and Taurine Metabolism	N-acetylmethionine	0.98	0.27
		hypotaurine	3.67	1.52
		taurine	0.04	4.80
	Creatine Metabolism	guanidinoacetate	0.46	1.33
		creatine	0.17	0.37
		creatinine	0.28	0.28
	Glutathione Metabolism	glutathione, reduced (GSH)	1.49	0.29
		ophthalmate	0.06	0.71
Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	3-phosphoglycerate	1.12	0.02
		phosphoenolpyruvate (PEP)	1.55	0.02
		pyruvate	1.23	1.75
		lactate	1.26	1.91
		glycerate	1.06	0.25
	Disaccharides and Oligosaccharides	lactose	0.39	0.21
	Nucleotide Sugar	UDP-glucose	10.53	0.35
		UDP-galactose	8.74	0.20
Energy	TCA Cycle	citrate	1.06	0.11
		aconitate [cis or trans]	1.18	0.24
		alpha-ketoglutarate	1.16	0.52
		fumarate	1.81	0.37
		malate	1.61	0.20

Lipid	Fatty Acid Metabolism(Acyl Carnitine)	acetylcarnitine (C2)	0.07	0.31
		octanoylcarnitine (C8)	0.06	0.53
		stearoylcarnitine (C18)	0.38	1.04
	Carnitine Metabolism	carnitine	0.06	1.25
	Phospholipid Metabolism	choline	1.05	0.51
		glycerophosphorylcholine (GPC)	2.52	0.45
		glycerophosphoethanolamine	1.98	0.48
	Phosphatidylcholine (PC)	1-palmitoyl-2-stearoyl-GPC (16:0/18:0)	0.54	0.48
		1-palmitoyl-2-alpha-linolenoyl-GPC (16:0/18:3n3)	0.57	0.40
		1,2-dilinoleoyl-GPC (18:2/18:2)	0.50	0.43
		1-linoleoyl-2-linolenoyl-GPC (18:2/18:3)	0.41	0.49
	Phosphatidylethanolamine (PE)	1,2-dipalmitoyl-GPE (16:0/16:0)	0.59	0.62
		1-palmitoyl-2-arachidonoyl-GPE (16:0/20:4)	0.56	0.53
		1-oleoyl-2-arachidonoyl-GPE (18:1/20:4)	0.63	0.56
	Phosphatidylserine (PS)	1-palmitoyl-2-oleoyl-GPS (16:0/18:1)	0.78	0.62
		1-stearoyl-2-oleoyl-GPS (18:0/18:1)	0.74	0.44
	Phosphatidylinositol (PI)	1-palmitoyl-2-arachidonoyl-GPI (16:0/20:4)	0.58	0.69
		1-stearoyl-2-oleoyl-GPI (18:0/18:1)	1.53	0.49
		1-stearoyl-2-arachidonoyl-GPI (18:0/20:4)	0.56	0.78
	Diacylglycerol	diacylglycerol (12:0/18:1, 14:0/16:1, 16:0/14:1) [2]	0.76	0.59
		palmitoyl-palmitoyl-glycerol (16:0/16:0) [2]	1.02	0.43
		linoleoyl-arachidonoyl-glycerol (18:2/20:4) [1]	1.00	0.31
		linoleoyl-arachidonoyl-glycerol (18:2/20:4) [2]	0.70	0.18
	Sphingolipid Metabolism	N-palmitoyl-sphinganine (d18:0/16:0)	2.96	1.26
		N-palmitoyl-sphingadienine (d18:2/16:0)	0.34	1.15
		tricosanoyl sphingomyelin (d18:1/23:0)	0.68	0.53
		sphingomyelin (d18:0/18:0, d19:0/17:0)	9.99	0.55
		hexadecasphingosine (d16:1)	0.20	1.03
		N-palmitoyl-heptadecasphingosine (d17:1/16:0)	0.37	1.01
	Ceramides	N-palmitoyl-sphingosine (d18:1/16:0)	0.46	1.19
		N-stearoyl-sphingosine (d18:1/18:0)	0.40	1.07
		ceramide (d18:1/17:0, d17:1/18:0)	0.34	1.08

Table 1: List of biochemical components detected in cumulus cells.
Fold changes in component concentration were calculated for cumulus cells after 8h maturation versus GV stage cumulus cells (0h), and cumulus cells matured for 23h versus 8h. Cells marked in red indicate a significant increase, and those marked in blue a significant decrease.**t of biochemical components detected in cumulus cells.**
Fold changes in component concentration were calculated for cumulus cells after 8h maturation versus GV stage cumulus cells (0h), and cumulus cells matured for 23h versus 8h. Cells marked in red indicate a significant increase, and those marked in blue a significant decrease.

the reaction of guanidinoacetate, concentrations of which were not changed after 8h of maturation, with S-adenosyl methionine. Creatine either reacts with ATP to yield the energy buffer, creatine phosphate (not detected), or is metabolised to the breakdown product creatinine, which was increased 22-fold in maturation medium after 8h. In contrast to the levels of other biochemical components, a ten-fold enrichment of urea was detected, with much of the increase taking place in the second part of maturation. Overall, these data indicate an increase in amino acid synthesis and/or protein breakdown during IVM. If protein breakdown occurs this would not be due to dying cumulus cells as indicated by the viability assay.

Carbohydrate Metabolism

Glucose, lactose, and pyruvate are considered important supplements to oocyte maturation medium [17]. Glucose in the medium is consumed by the cumulus cells and converted into pyruvate, the preferred energy substrate of the oocyte.

During the first 8h of maturation, concentrations of UDP-glucose and UDP-galactose in the cumulus cells increased more than other components of carbohydrate metabolism (Table S1); indeed, levels of detectable components of the carbohydrate categories glycolysis, gluconeogenesis, and pyruvate metabolism did not change during this period (Table 1). During anaerobic glycolysis, pyruvate is converted into lactate, the concentration of which increased significantly in cumulus cells during the final 15h of maturation. During the entire 23h IVM, the levels of most biochemical components of carbohydrate metabolism pathways, such as phosphoenolpyruvate (PEP) and 3-phosphoglycerate, decreased in cumulus cells (Table 1).

The concentration of glucose in maturation medium decreased significantly over time of IVM, indicating that it was consumed by the COCs. Surprisingly, during this period no significant change in the concentrations of lactate was detected in cumulus cells, whereas in the maturation medium the biggest increase among carbohydrate pathway components observed was for lactate; after 8h, lactate concentrations had increased 667-fold (8h versus 0h) and after 23h by 1227-fold (23h versus 0h; Table S2). In addition, increased levels of mannitol/sorbitol and ribitol were detected after 8h. Pyruvate is the end product of glycolysis and was not present in unconditioned medium, but it was produced by the COCs and secreted into the maturation medium. An increased pyruvate concentration in the conditioned maturation medium was therefore detected during the second part of IVM (8-23h) (Table 2).

Glucose is a central molecule in many metabolic pathways. Compared with cumulus cells collected at 0h, the concentration of glucose did not change in cumulus cells after 8h or 23h of maturation, suggesting that glucose was immediately processed by cumulus cells to lactate or pyruvate, the preferred energy substrates for the oocyte.

Energy Metabolism

Pyruvate is the end product of glycolysis and can enter the tricarboxylic acid cycle, an important route for ATP production. Cumulus cells matured for 8h showed an increase in the concentration of fumarate, an intermediate of the tricarboxylic acid cycle, whereas the concentrations of other intermediates were not changed. By contrast, in cumulus cells matured for 23h, the concentrations of citrate, aconitate, α-ketoglutarate, succinate, fumarate, and malate decreased (Table S1).

In conditioned medium, the concentrations of citrate, α-ketoglutarate, succinate, and fumarate were significantly increased after 8h and 23h. Indeed, the concentrations of nearly all detectable components of the tricarboxylic acid cycle increased in maturation medium during IVM. In particular, α-ketoglutarate and succinate accumulated in the maturation medium during the first 8h, whereas during the second part of maturation the concentrations of fumarate and malate increased (Table 2).

The decrease in tricarboxylic acid cycle metabolites in the cumulus cells indicates reduced pyruvate oxidation in the mitochondria of those cells. Additionally, intermediates of the tricarboxylic acid cycle were secreted into the maturation medium, where they might be available for other reactions in follicular cells; for example, malate can be used for amino acid synthesis.

Lipid metabolism

The main lipid metabolic pathways can be subdivided into different specific systems, such as the phosphatidylcholine and phosphatidylinositol pathways. In general, decreased levels of biochemical components of phospholipid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, diacylglycerol, sphingolipid, and ceramide metabolism were detected in cumulus cells (Table S1, Figure S2). During maturation, concentrations of most of the lipids decreased in cumulus cells by 8h and 23h, among which components of the phosphatidylcholine and ceramide pathways showed a more prominent decline during the first 8h. The biochemical

Table 2

Maturation medium			Fold change	
Pathway	Sub Pathway	Biochemical Name	8h vs. 0h	23h vs. 8h
Amino Acid	Glycine, Serine, and Threonine Metabolism	glycine	1.05	1.13
		N-acetylglycine	1.06	2.30
		betaine	6.80	0.81
		serine	0.79	0.61
		N-acetylserine	1.68	2.78
		N-acetylthreonine	1.00	2.75
	Glutamate Metabolism	glutamate	0.99	1.13
		N-acetylglutamate	2.92	2.43
		N-acetylglutamine	4.14	1.46
		pyroglutamine	0.42	1.35
		S-1-pyrroline-5-carboxylate	0.68	1.17
	Leucine, Isoleucine, and Valine Metabolism	N-acetylleucine	1.11	1.91
		4-methyl-2-oxopentanoate	7.55	2.28
		3-methyl-2-oxovalerate	4.92	2.66
		3-methyl-2-oxobutyrate	1.69	3.33
		3-hydroxyisobutyrate	1.08	5.58
	Methionine, Cysteine, SAM, and Taurine Metabolism	N-acetylmethionine	2.77	5.28
		methionine sulfoxide	0.45	1.06
		hypotaurine	35.65	2.54
		taurine	1.75	0.84
	Urea cycle; Arginine and Proline Metabolism	urea	1.02	9.51
		ornithine	13.39	2.88
		2-oxoarginine	1.81	1.38
		dimethylarginine (SDMA + ADMA)	6.92	3.63
		trans-4-hydroxyproline	0.98	1.05
	Creatine Metabolism	guanidinoacetate	1.18	3.16
		creatine	427.32	1.21
		creatinine	21.62	0.95
	Glutathione Metabolism	cysteine-glutathione disulfide	1.32	2.50
		2-hydroxybutyrate/2-hydroxyisobutyrate	1.84	2.15
Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	glucose	0.59	0.17
		pyruvate	1.15	3.19
		lactate	667.34	1.84
		glycerate	1.43	1.38
	Pentose Metabolism	ribose	0.78	1.09
		ribitol	4.08	1.70
		ribonate	2.40	1.07
		arabitol/xylitol	1.65	2.02
	Fructose, Mannose, and Galactose Metabolism	fructose	0.52	1.04
		mannitol/sorbitol	5.90	1.39
		mannose	1.03	0.75
	Aminosugar Metabolism	erythronate	1.22	1.95
		N-acetylglucosamine/N-acetylgalactosamine	1.00	4.52

Energy	TCA Cycle	citrate	3.01	1.80
		aconitate [cis or trans]	1.00	1.96
		alpha-ketoglutarate	11.53	1.88
		succinate	3.66	1.49
		fumarate	1.58	2.90
		malate	1.70	4.17
Lipid	Medium Chain Fatty Acid	heptanoate (7:0)	0.64	0.77
	Fatty Acid, Dicarboxylate	glutarate (pentanedioate)	0.68	1.81
		2-hydroxyglutarate	1.78	1.82
	Carnitine Metabolism	carnitine	1.77	0.89
	Inositol Metabolism	myo-inositol	11.70	0.58
	Phospholipid Metabolism	choline	0.36	0.88
		choline phosphate	75.06	1.40
		glycerophosphorylcholine (GPC)	1.21	1.56
		glycerophosphoethanolamine	1.21	1.62
		glycerophosphoinositol	1.27	2.49
	Glycerolipid Metabolism	glycerol	6.89	1.47
		glycerophosphoglycerol	4.13	2.45
	Mevalonate Metabolism	3-hydroxy-3-methylglutarate	4.22	4.22

Table 2: List of biochemical components detected in cumulus-oocyte-complex-conditioned medium. Fold changes in biochemical component concentration were calculated for medium conditioned for 8h versus unconditioned medium (0h) and for medium conditioned for 23h versus 8h. Cells highlighted in red exhibited a significant increase, and those highlighted in blue a significant decrease.

components with the lowest fold change in cumulus cells were acetylcarnitine, octanylcarnitine, hexanoylcarnitine, and carnitine. Over the 23h of maturation, the concentrations of various components of phosphatidylcholine, phosphatidylethanolamine, and sphingolipid metabolism decreased in cumulus cells (Table S1).

In COC-conditioned medium, increased levels of carnitine, myo-inositol, cholinephosphate, and glycerol were detected after 8h of maturation. The comparison between 8h and 23h demonstrated a decrease in the concentrations of myo-inositol, indicating that myo-inositol was consumed during the last 15h of maturation (Table 2, Figure S2). Carnitine is an important factor during β-oxidation, and its concentrations in cumulus cells decreased after 8h of maturation (16.7-times); the levels of its derivatives also decreased sharply in cumulus cells during the first 8h of maturation. Furthermore, during 8h of conditioning the amount of carnitine in the maturation medium increased 1.8-fold.

These data show that, in cumulus cells, phospholipids are broken down to yield phospholipid precursors which are excreted, possibly for use by the oocyte.

Carnitine and creatine supplementation

Carnitine and creatine were among the biochemical components that were elevated in COC-conditioned medium after 8h. In order to better understand their functions, oocytes were matured in the presence of these components of lipid metabolism (carnitine) or the amino acid pathway (creatine). Oocytes were denuded and matured for 23h or denuded 8h after the onset of maturation and subsequently placed into fresh maturation medium supplemented with carnitine or creatine (Figure 1a).

The proportion of oocytes that had cleaved 5 days after fertilization was decreased for oocytes denuded after 8h of maturation and placed into fresh maturation medium compared to control COCs; however, supplementation of the maturation medium with carnitine resulted in cleavage rates similar to the control (Figure 4a). Most importantly, denuded oocytes matured in maturation medium supplemented with 2.5 or 10 mM carnitine yielded better cleavage rates than denuded oocytes matured without carnitine supplementation (Figure 4b). Percentages of oocytes that developed to blastocysts after fertilization were also increased after carnitine supplementation, compared to

denuded oocytes placed into fresh maturation medium after 8h of maturation (Figure 4c) and oocytes matured for 23h in the absence of cumulus cells (Figure 4d). Combined, these data demonstrate the importance of carnitine for the acquisition of developmental competence by a denuded oocyte during *in vitro* maturation.

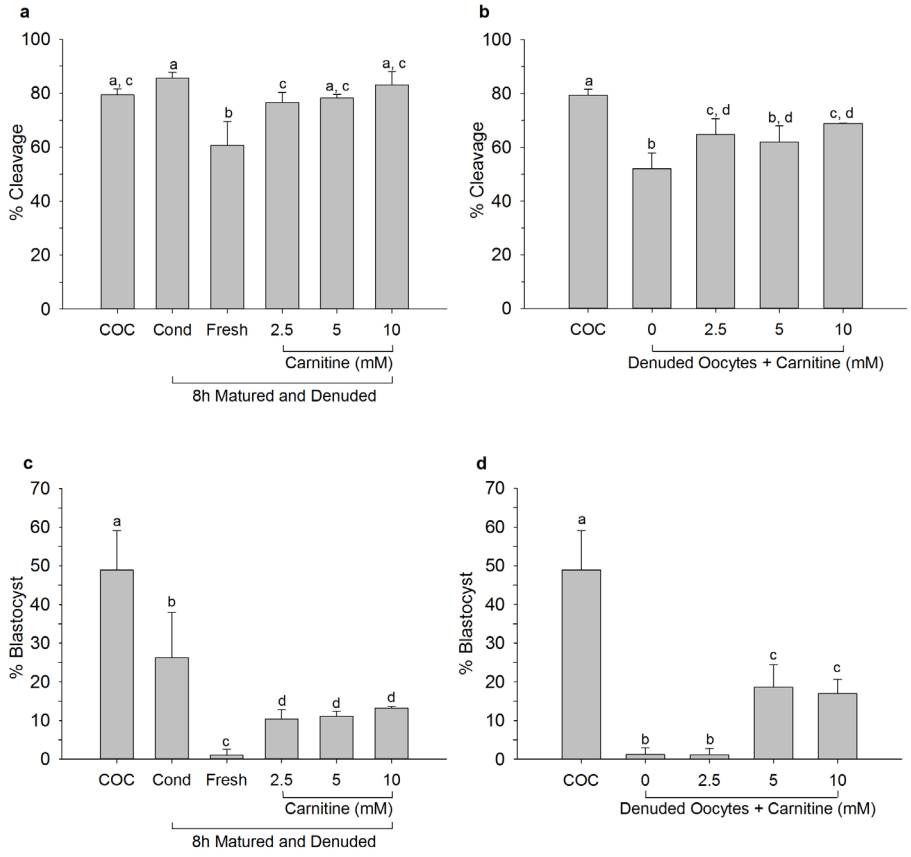

Figure 4: Cleavage and blastocyst formation with carnitine supplementation
Cleavage of oocytes (a, b) and blastocyst formation of cleaved oocytes (c, d) matured in carnitine supplemented medium. Oocytes were matured as intact COCs for 23h (COC) or as intact COCs for 8h before being denuded and placed back in their own conditioned medium (Cond), or transferred to fresh maturation medium (Fresh) or to fresh maturation medium supplemented with varying concentrations of carnitine. Denuded oocytes were matured for 23h (Oocytes) in conventional medium or for 23h in carnitine-supplemented medium. Data are presented as mean ± SD. Data points labelled with the same letter did not differ significantly (N=3; P<0.05).

Success of cleavage for oocytes matured for 8h, denuded and placed into creatine supplemented maturation medium were the same as those for similar oocytes placed in fresh medium (Figure 5a). Similarly, creatine supplementation of maturation medium did not significantly alter the percentage of oocytes that cleaved after maturation for 23h without cumulus cells (Figure 5b). Blastocyst formation for oocytes denuded after 8h or matured for the entire 23h without cumulus cells was also not improved by creatine supplementation (Figure 5c, d). These data suggest that creatine alone does not affect the developmental competence of the oocyte during maturation.

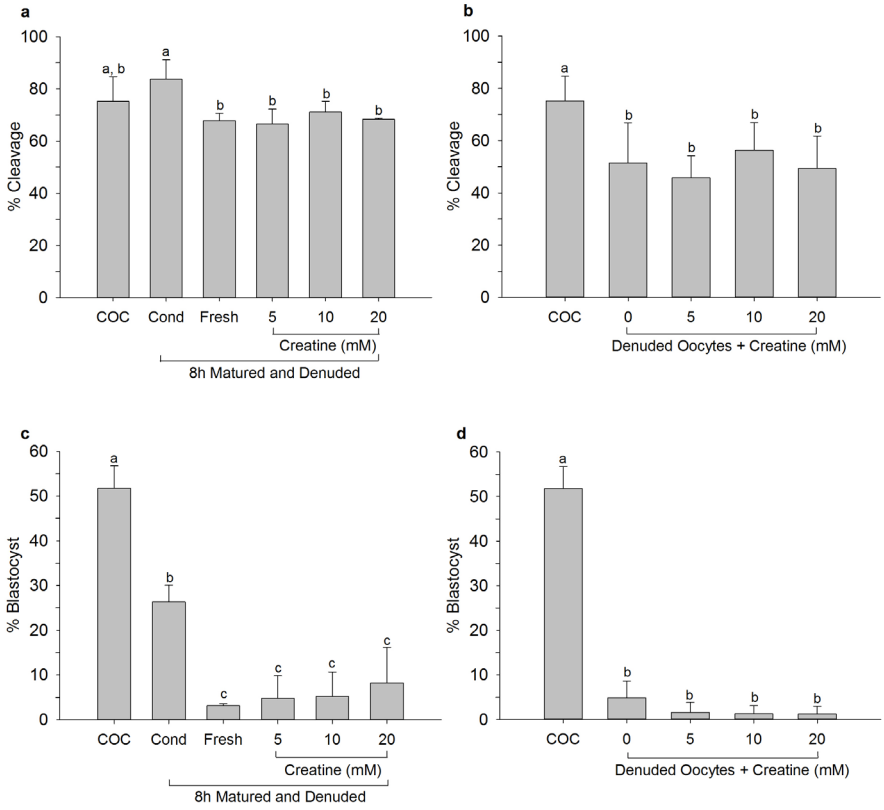

Figure 5: Cleavage and blastocyst formation with creatine supplementation
Cleavage of oocytes (a, b) and blastocyst formation of cleaved oocytes (c, d) matured in creatine supplemented medium. Oocytes were matured as intact COCs for 23h (COC) or as intact COCs for 8h before being denuded and placed back in their own conditioned medium (Cond), transferred to fresh maturation medium (Fresh) or into fresh maturation medium supplemented with varying concentrations of creatine. Denuded oocytes were matured for 23h (Oocytes) or for 23h in creatine-supplemented medium. Data are presented as mean ± SD. Data points labelled with the same letter did not differ significantly (N=3; $P<0.05$).

Discussion

Oocyte developmental competence is influenced by direct contact with cumulus cells, and by the microenvironment created by the cumulus-oocyte-complex during maturation. The importance of cumulus cells for oocyte maturation and the acquisition of developmental competence has been reported previously [1, 5]. Here, we examined the metabolic profile of cumulus cells at different time points during maturation, and that of COC-conditioned medium. The viability of the cells during the whole maturation period was demonstrated by an AlamarBlue assay.

Our results show that, during the first 8h of maturation, a microenvironment is created that supports oocyte developmental competence. If oocytes were matured for 8h within intact COCs, denuded and placed back into their conditioned medium, 26% developed to blastocysts, compared to only 2% if the oocytes were placed back into fresh (non-conditioned) maturation medium. This demonstrates that COCs secrete factors during the first 8h of maturation that are beneficial for oocyte acquisition of developmental competence during the following 15h of maturation.

To identify candidate metabolites that improve oocyte maturation, a UPLC-MS/MS analysis was performed. It transpired that the concentrations of 77 different biochemical components increased and 181 decreased in the cumulus cells (Figure 3a, b), whereas the concentrations of 113 metabolites increased and 27 decreased (Figure 3b, d) in the maturation medium during all analysed time points. In total more compound species were detected in cumulus cells than in conditioned medium. Since detection levels were similar between media and cells this indicates that indeed more components were present in cumulus cell than secreted into the medium. Most probably, the majority of changes in metabolite concentrations result from the general metabolic activity of the cumulus cells, nevertheless the IVM experiments demonstrate that cumulus cells also secrete factors that assist the oocyte to successfully complete the maturation process. In theory, decreased component levels in condition medium could also have resulted from degradation of metabolites in the medium, independent of the presence of the oocyte and cumulus cells between 8 and 23h of maturation.

The increase in concentration for most of the amino acids in both the cumulus cells and medium probably reflects normal metabolism. One exception was the decrease in cellular serine concentrations during the final 15h of maturation, and a decrease in concentrations in the conditioned medium throughout. Cancer cells deprived of serine have been reported to undergo oxidative stress [18]. It is possible that oocytes similarly require serine from the environment to reduce oxidative stress by channelling depleted serine stores to glutathione synthesis, as cancer cells do [19]. Alternatively, serine may be used for one-carbon metabolism [18] to generate one-carbon units for nucleotide synthesis, e.g. glycine or α-ketoglutarate.

In bovine oocytes, oxidative metabolism is the major mechanism responsible for energy production [20]. Bovine cumulus cells exhibit high alanine aminotransferase and aspartate aminotransferase activity, which is presumably linked to amino acid synthesis [21]. In addition it has been suggested that aspartate has an important role in oocyte energy metabolism, because it can be converted into oxaloacetate for use in the tricarboxylic acid cycle [21]. Indeed, our results show a significant increase in aspartate concentrations in cumulus cells at all time points tested (see also Table S1); however, concentrations did not change in the surrounding media during maturation, suggesting that aspartate is used directly for energy production in the cumulus cells or is transferred to the oocyte. Supplementation of maturation media with aspartate or alanine has been reported to improve the maturation of denuded oocytes, indicating their importance as oxidative substrates for the oocyte [21] or in the synthesis of protein factors involved in meiotic maturation. However, in our study no IVM-related changes in alanine concentrations were detected in cumulus cells, and only a small increase between 8h and 23h of maturation was detected in conditioned medium.

Glucose is metabolized to pyruvate during glycolysis, and pyruvate is in turn partly converted into lactate via anaerobic glycolysis. It has been demonstrated that cumulus cells can metabolize glucose into pyruvate, and deliver the latter to the oocyte [22, 23]. Pyruvate can be directly transferred via gap junctions in the transzonal processes from the cumulus cell to the oocyte, but it can also be secreted by the cumulus cells and taken up from the surrounding medium by the oocyte [24]. Indeed, in conditioned medium the concentrations of both pyruvate and lactate increased, while glucose concentrations decreased by 23h of maturation. In particular, the lactate concentration of maturation medium increased 667-fold after 8h IVM, whereas in cumulus cells an

increase was detected during the final 15h of maturation. During the first 8h of maturation, there was no significant change in pyruvate in the cumulus cells or maturation medium, suggesting that during the first 8h pyruvate is channelled to additional metabolic pathways such as the tricarboxylic acid cycle. Indeed, a significant increase in tricarboxylic acid cycle metabolites, such as citrate and α-ketoglutarate were detected. Taken together these data indicate that glucose is utilized for pyruvate production, especially during the first 8h of maturation, and that the pyruvate is taken up by the oocyte as an energy substrate. Alternatively, pyruvate could be converted into lactate. Indeed, oocytes are rich in members of the SLC16A (MCT) family of monocarboxylic acid transporters [25] that can transport lactate. In addition, supplementation of maturation medium with lactate and NAD has been reported to improve maturation of denuded bovine oocytes [26].

Interestingly, between 8h and 23h of IVM the concentrations of almost all detected components of the tricarboxylic acid cycle decreased in cumulus cells. By contrast, the medium showed a temporally related increase in these components. Other studies have reported that culture of denuded bovine oocytes with intermediates of the tricarboxylic acid cycle, oxaloacetate or malate, in combination with NAD, a coenzyme important for the redox status of the cell, resulted in a significant improvement in the proportion reaching MII [21]. By contrast, no such improvement was detected when oocytes were cultured as COCs [21]. Together these data suggest that biochemical processes in cumulus cells can influence the redox state of the oocyte. This is important for further development, because excess reactive oxygen species generation compromises the oocyte's developmental competence.

Increased concentrations of nucleotide sugars, including UDP-glucose, were detected in cumulus cells, especially after the first 8h of maturation. UDP-glucose is involved in synthesis of hyaluronan, an element of the cumulus cell matrix, suggesting that UDP-glucose is involved in cumulus cell matrix changes during maturation. Indeed, an increase in UDP-glucose and UDP-galactose was evident only during the first 8h of maturation, the critical period for cumulus expansion.

Lipids were mostly detected in cumulus cells and, in general, their concentrations decreased during maturation. The concentrations of some components of phospholipid metabolism, like choline phosphate, glycerophosphoethanolamine, and glycerophosphoinositol increased in the

medium, however, suggesting that they were generated by the COC. Lipid droplets have also been found in cumulus cells and β-oxidation seems to take place predominantly in the cumulus cells [27, 28]. Energy production, in terms of ATP generation, is much higher for β-oxidation than glycolysis [27], but it was not known whether fatty acids from the follicular fluid were used to generate ATP. Supplementation of maturation medium with linolenic acid has been reported to have a positive effect on oocyte maturation and embryo development [29] and was able to neutralise the detrimental effects of palmitic acid and stearic acid on *in vitro* maturation [30]. Furthermore, cumulus cells regulate the transport of fatty acids to the oocyte, after exposure to elevated free fatty acids cumulus cells stored these lipids [31]. Cholesterol biosynthesis also requires oocyte-cumulus cell cooperation [32], since transcripts important for cholesterol synthesis pathways have been found in cumulus cells, but not in oocytes [33, 34]. This indicates that, in terms of energy production, lipids are important during oocyte maturation and establishment of a developmentally competent oocyte. The concentrations of various lipid classes, such as phosphatidylcholine and phosphatidylethanolamine, decreased in cumulus cells during maturation, however they were not detected in the maturation medium, suggesting metabolic cooperation in lipid metabolism between the cumulus cells and the oocyte. Whether the changes observed in the *in vitro* matured cumulus cells also apply to those from *in vivo* matured COCs remains to be established.

Of the components that decreased in cumulus cells and increased in medium, the influence of carnitine and creatine were tested further. Creatine was chosen for its impressive concentration increase in the medium within 8h. Carnitine was chosen because its concentration also increased in the medium but more importantly because in cumulus cells the levels of carnitine metabolites (acetylcarnitine, hexanoylcarnitine and octanoylcarnitine) decreased, suggesting that they are further metabolised and this pathway is important for energy production.

While creatine supplementation had no effect, supplementation with carnitine improved the developmental competence of denuded oocytes. This effect was also detected, if oocytes having a low developmentally potential [35] or juvenile oocytes [36] were cultured with L-carnitine. Pig oocytes matured in glucose free maturation medium supplemented with carnitine, showed accelerated nuclear maturation and an enhanced ability to reach the MII stage [37]. A study in camels also showed a positive effect of carnitine supplementation on

oocyte nuclear maturation, as well as on the developmental potential of the oocytes [38]. All of which supports the hypothesis that fatty acid metabolism is important during oocyte maturation. In addition, carnitine has antioxidant activity which has been reported to protect the DNA against fragmentation due to reactive oxygen species generation [37, 39]. An example for antioxidants is glutathione, whose levels were increased after carnitine supplementation [40, 41].

Creatine supplementation did not improve blastocyst production. This was surprising because creatine can enter the adenosine salvage pathway [42] to provide ATP for the oocytes and the enzyme creatine kinase was associated with ATP supply during spindle formation in 2- and 4-cell mouse embryos [43]. Possibly, energy storage and transportation is more established during the later stages of maturation, since the phosphocreatine concentration in cumulus cells was decreased after 23h of maturation.

In summary, numerous metabolic processes occur in the cumulus cells during the first 8h of IVM that help to create a beneficial microenvironment for the maturing oocyte, which needs to store energy and other small molecules to ensure that it remains metabolically active until embryonic genome activation takes place.

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Supplementary Information

Can be found here:
https://drive.google.com/drive/folders/1J0mUYfMk2op97GaLyg4lFLaLl0V4y2_3?usp=sharing


5

Exposure to elevated glucose concentrations alters the metabolomic profile of bovine blastocysts

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Abstract

Chronically high blood glucose concentrations are a characteristic of diabetes mellitus. Maternal diabetes affects the metabolism of early embryos and can cause a delay in development. To mimic maternal diabetes, bovine *in vitro* fertilization and embryo culture were performed in culture medium containing 3 mM glucose. The metabolomic profile of resulting day 8 blastocysts was analysed by UPLC-MS/MS, and compared to that of blastocysts cultured in control conditions. Elevated glucose concentrations stimulated an increase in glycolysis and activity of the hexosamine pathway, which is involved in protein glycosylation. However, components of the tricarboxylic acid cycle, such as citrate and α-ketoglutarate, were reduced in glucose stimulated blastocysts, suggesting that energy production from pyruvate was inefficient. On the other hand, activity of the polyol pathway, an alternative route to energy generation, was increased. In short, cattle embryos exposed to elevated glucose concentrations during early development showed changes in their metabolomic profile consistent with the expectations of exposure to diabetic conditions.

Introduction

In diabetic patients, glucose utilization and storage is not regulated properly, resulting in hyperglycemia and glycosuria. Of the two major types of diabetes, type 1 is an autoimmune disease that generally onsets during childhood, whereas type 2 diabetes is the result of insulin resistance, and is mostly triggered by an unhealthy lifestyle, including poor nutrition and physical inactivity. Diabetes is an increasingly prevalent disease; in 2015, it was estimated that 415 million people worldwide suffered from some form of diabetes, and it is expected that this will increase to 642 million people by 2040, largely as a result of an increasing incidence of insulin resistance linked to obesity [1]. In addition to diabetes-related pathology in the primary patient, there is an increased risk of gestational complications, including spontaneous abortion and the development of metabolic diseases in the children of mothers who are, or become, diabetic during pregnancy [2]. Gestational diabetes occurs spontaneously and is triggered by pregnancy-related changes in the endocrine environment [3], but disappears after birth of the child. However, women that develop gestational diabetes, and their children, have a higher risk of developing type 2 diabetes later in life [1]. In 2015, it was estimated that 20.9 million children were born to mothers suffering from hyperglycemia during pregnancy [1].

Although blood glucose concentrations fluctuate markedly in healthy individuals depending primarily on the time and composition of their last meal, they are homeostatically regulated by insulin, glucagon and other catabolic hormones, such that they return to normoglycemic resting values between approximately 4 and 6 mM. By contrast, the blood glucose concentrations of diabetic patients are chronically elevated to approximately 12-19 mM, primarily as a result of either inadequate insulin production (type 1 diabetes) or insensitivity to the actions of insulin (type 2 diabetes). In women, high blood glucose concentrations are reflected in reproductive tract fluids, and may therefore affect oocyte maturation and early embryo development [4]. Indeed, careful regulation of the metabolic status of women suffering from diabetes during pregnancy has been shown to decrease the incidence of fetal abnormalities [5]. How, and to what extent, high glucose concentrations affect pre-implantation development is less well understood, primarily because of the relative inaccessibility of affected early embryos. To investigate the possible effects of maternal diabetes on oocytes or early embryos, various animal models have therefore been developed. A diabetic

mouse model demonstrated that oocytes from hyperglycemic animals are smaller and exhibit delayed meiotic maturation when compared to oocytes from control mice [6, 7]. In addition, both oocyte developmental competence [8–10] and early embryonic development are compromised by hyperglycemia in diabetic animal models including the mouse, rat, rabbit, sheep and cow [4, 11–15]. While most diabetes studies have been performed in rodent models, using bovine blastocysts has several advantages for extrapolation to human development. The main advantages of bovine oocytes include accessibility and avoiding the use of experimental animals, since cow ovaries can be obtained as a by-product from slaughterhouses. Furthermore, like man, the cow is a mono-ovulatory species with a similar time course of oogenesis and folliculogenesis, making the cow a good animal model for human reproduction [16, 17]. In addition, germinal vesicle breakdown during maturation requires protein synthesis in human and bovine oocytes [18, 19], which is not needed in the mouse [20].

During oocyte maturation, the cumulus cells that surround the oocyte are glycolytically active; they take up glucose and generate pyruvate, the preferred metabolic substrate of the oocyte [21–23] and preimplantation embryo [24, 25]. In addition, the pentose phosphate pathway is reported to play an important role in glucose utilization during meiotic maturation [26, 27] and early embryo development [28, 29]. Overall, it is thought that, before it enters the uterus, the embryo's main energy substrates are pyruvate and lactate, which are metabolized via oxidative phosphorylation. After arrival in the relatively anaerobic environment of the uterus, the embryo switches its primary mode of metabolism to glycolysis [30].

Exactly how high glucose concentrations affect the metabolism of the preimplantation embryo is not well understood. Here, we used bovine *in vitro* fertilization and embryo development in the presence of exogenous glucose to create an environment mimicking that expected in a diabetic mother, and compared the metabolomic profile of resulting blastocysts and the medium conditioned by embryo culture with those of glucose-free culture.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Collection of cumulus-oocyte complexes, in vitro maturation, fertilization and embryo culture

Cattle ovaries were collected from a local slaughterhouse (Gosschalk, Epe, The Netherlands) and transported to the laboratory in a thermos flask. On arrival at the laboratory, the ovaries were rinsed in tap water and maintained at 30°C in physiological saline (0.9%) containing 100 IU/ml penicillin and 100 µg/ml streptomycin. Cumulus-oocyte complexes (COCs) were then aspirated from small antral follicles (2-8 mm) using an 18-gauge needle connected via a 50 ml collection tube to a low-pressure aspiration pump. COCs with several cumulus cell layers were selected and, after washing in HEPES buffered M199 (Gibco BRL, Paisley, UK), were matured *in vitro* in groups of 50-60 COCs. All culture steps (maturation, fertilization and embryo culture) took place in 4-well culture plates (Nunc A/S, Roskilde, Denmark). To allow *in vitro* maturation, COCs were incubated for 23h in 500 µl $NaHCO_3$-buffered M199 (Gibco BRL), which contains 5.5 mM glucose, supplemented with 1% penicillin-streptomycin (Gibco BRL), 0.02 IU FSH/ml (Sioux Biochemical Inc., Sioux Centre IA, USA), 0.02 IU LH/ml (Sioux Biochemical Inc.), 7.7 µg/ml cysteamine and 10 ng/ml epidermal growth factor, at 39°C in a humidified atmosphere containing 5% CO_2.

In vitro fertilization was performed as described previously [31, 32]. In brief, fertilization medium which does not contain glucose but instead contains sodium pyruvate as an energy source, was supplemented with 1.8 IU/ml heparin, 20 µM d-penicillamine, 10 µM hypotaurine, and 1 µM epinephrine. Frozen-thawed spermatozoa from an *in vitro* fertility proven bull was washed through a Percoll gradient and used at a concentration of 1×10^6 sperm cells/ml. After sperm-oocyte co-incubation for 18-22h at 39°C in a humidified atmosphere containing 5% CO_2 and 7% O_2, presumptive zygotes were denuded of their cumulus investment by vortexing for 3 min, and transferred to synthetic oviductal fluid (SOF) [33] for further culture. Assessment of presumptive embryos for cleavage was performed on day 5 of culture, and only cleaved embryos were transferred to fresh SOF and cultured for an additional 3 days (until day 8), when blastocyst development was assessed. Fertilization medium and SOF containing 0 (control) or 3 mM glucose were used for the metabolomic study.

Collection of blastocysts and culture medium for metabolomic analysis

Day 8 blastocysts were washed in PBS, snap frozen in liquid nitrogen and stored at -80°C until processing for metabolomic analysis. In total, 3 samples from each condition, containing between 538 and 590 blastocysts per sample were analysed. Medium (500 µl) was conditioned by incubation of at least 50 embryos with more than eight-cells from day 5 to day 8 in SOF medium, either without glucose (control) or supplemented with 3 mM glucose.

Sample preparation and metabolomic analysis

Sample preparation and ultra-high performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS) were performed as described previously [34] by Metabolon Inc. (Durham, NC USA). In brief, 4 analysing methods were used, two separate reverse phase (RP) UPLC-MS/MS methods employing positive ion mode electrospray ionization, one analysis by RP/UPLC-MS/MS with negative ion mode electrospray ionization, and one analysis by hydrophilic interaction chromatography (HILIC)/UPLC-MS/MS with negative ion mode electrospray ionization. Raw data were extracted, peak-identified and QC-processed using Metabolon's proprietary hardware and software. Measurements for blastocysts were normalized against total protein concentration. In the case of missing values, imputation was used to calculate the fold change (marked with an asterisk in the supplementary tables).

Statistical analysis

Statistical analysis of the success of blastocyst development in medium with different glucose concentrations was performed using SigmaPlot software (Systat software Inc., San Jose, CA, USA), these data are expressed as means ± SD. To compare the percentage of blastocysts between treatment groups, after testing for normality of distribution, an analysis of variance (ANOVA) was performed followed by a post-hoc Bonferroni test. Welch's two-sample t-test was used to compare metabolite concentrations between 0 and 3mM glucose conditions. A P-value < 0.05 was considered to be statistically significant.

Results

In order to determine tolerance of bovine embryos to glucose, bovine oocytes were fertilized and cultured to the blastocyst stage in the presence of exogenous glucose. The percentages of oocytes that developed to blastocysts decreased significantly at glucose concentrations as low as 3 mM (Figure 1). To further understand how blastocysts that developed in the presence of elevated glucose concentrations differed to those that developed in the absence of exogenous glucose, the metabolomic profiles of the blastocysts were determined by mass spectrometry. In total, 1719 control blastocysts and 1698 glucose-stimulated (3 mM) blastocysts were divided into three groups for metabolite identification. In addition, the medium in which embryos had been cultured from days 5 to 8 was analysed for metabolite concentrations.

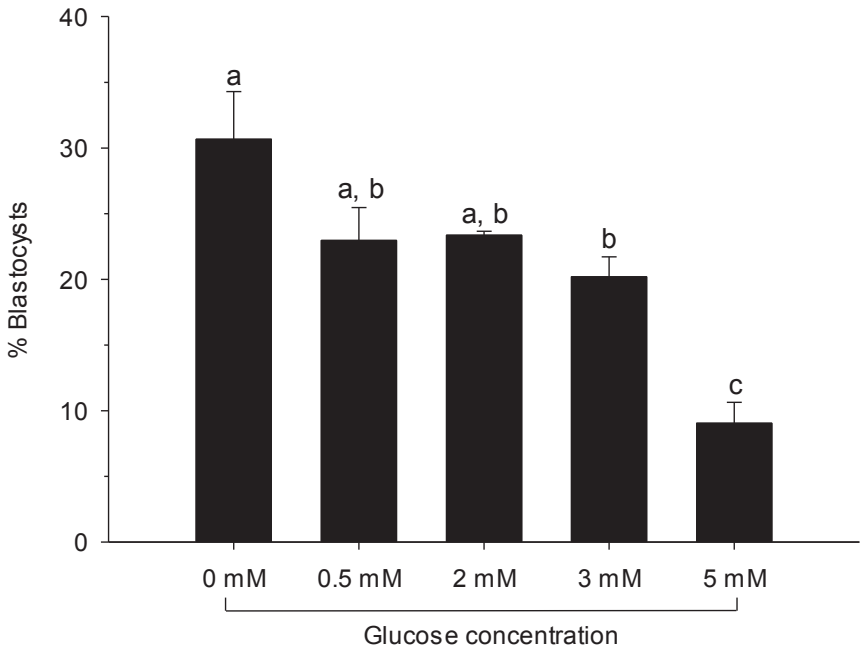

Figure 1: Effect of glucose on bovine blastocyst development.
Fertilization and embryo culture was performed in culture medium without supplementary glucose (control) and in medium containing final glucose concentrations as indicated. Blastocyst development was evaluated on day 8 of post-fertilization culture. Data are depicted as mean ± SD; columns with different letters differ significantly.

The metabolomic profiles revealed 22 biochemical constituents that increased and 41 that decreased significantly in concentration, in glucose-stimulated compared to control blastocysts. The comparison of conditioned media from the two types of culture showed significantly altered concentrations of 18 biochemical compounds, 11 of which were increased and 7 decreased when blastocysts were cultured in medium containing elevated glucose concentrations. As expected, the intraembryonic glucose concentration increased markedly when embryos were cultured in the presence of 3 mM glucose (Figure 2a, Supplementary Table 1). Intracellular concentrations of 3-phosphoglycerate and phosphoenolpyruvate (Figure 2b, c, Supplementary Table 1) were also increased significantly, indicating enhanced glycolytic activity; however, intracellular concentrations of pyruvate and lactate were not altered (Figure 2d, e, Supplemental Table 1). Embryonic mannitol/sorbitol and fructose concentrations were however increased, indicating glucose metabolism via the polyol pathway (Supplementary Table 1). Surprisingly, given that sorbitol does not diffuse efficiently through the plasma membrane, the concentrations of sorbitol and fructose were also increased in the medium conditioned by glucose-stimulated embryos (Figure 3a, b, Supplemental Table 2). And while the pyruvate concentrations in the conditioned medium tended to increase (Figure 3c, Supplementary Table 2), the increase did not reach statistical significance ($P=0.09$). Increased intracellular concentrations of N-acetylglucosamine, N-acetyl-glucosamine 1-phosphate, UDP-N-acetylglucosamine/galactosamine, and N-acetylneuraminate (Supplementary Table 1) indicate increased hexosamine pathway activity in the glucose-stimulated blastocysts. Sedoheptulose, an intermediate of the pentose phosphate pathway, was detected in glucose-stimulated blastocysts only. A reduced concentration of oxidized glutathione was also detected in glucose-stimulated blastocysts (Figure 2f).

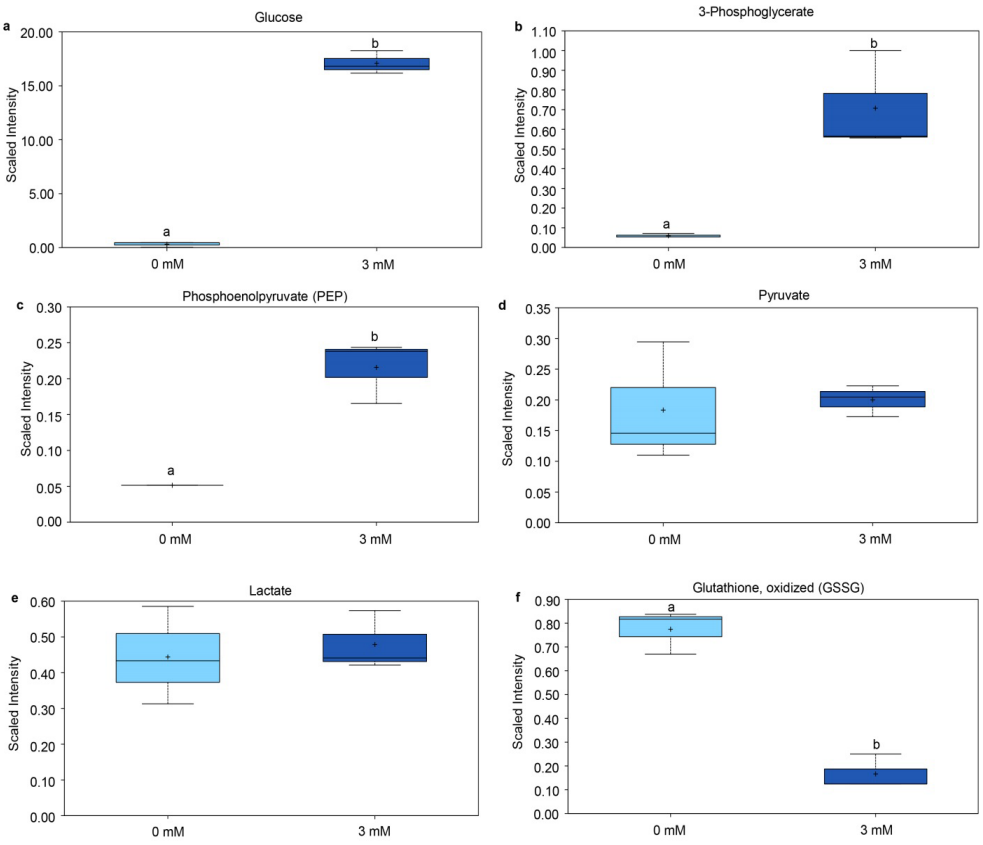

Figure 2: Selected biochemicals detected in bovine blastocysts.
Box and whisker plots for biochemical components detected in day 8 blastocysts produced in medium in the presence or absence (control) of 3 mM glucose. a) glucose, b) 3-phosphoglycerate, c) phosphoenolpyruvate, d) pyruvate, e) lactate and f) oxidized glutathione (GSSG). The upper whiskers represent the maximum, and the lower whiskers the minimum values. The plus-signs indicate the mean values, while the median values are represented by a black line within the boxes. Boxes with different letters differ significantly. Light blue – d8 blastocysts without supplementary glucose; blue – d8 blastocysts cultured in the presence of 3 mM glucose.

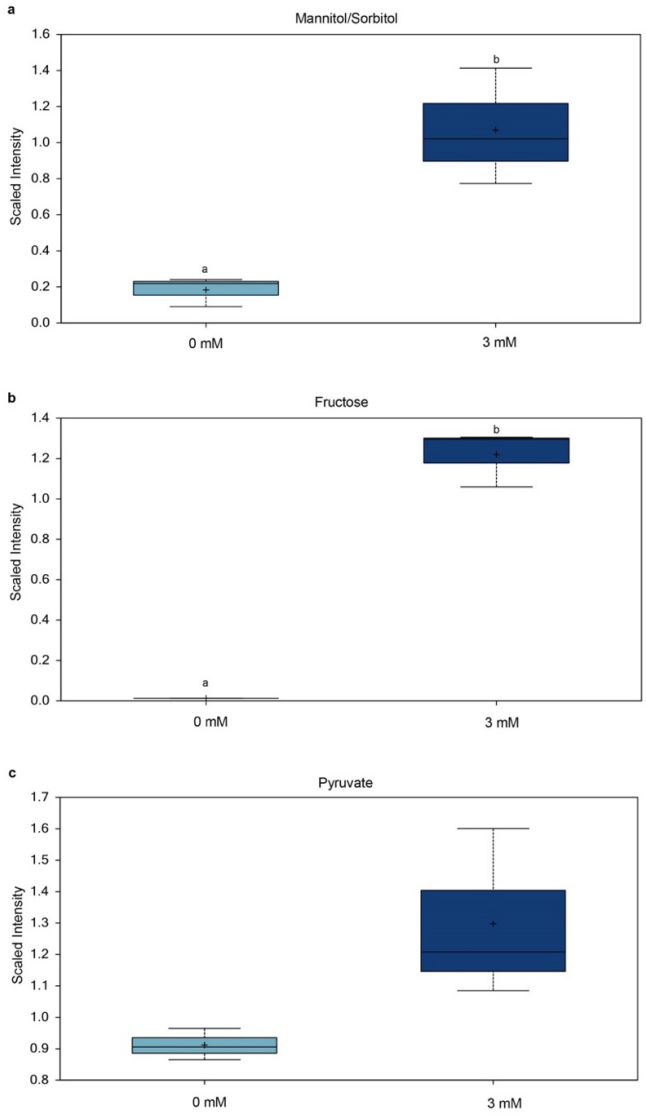

Figure 3: Selected biochemicals detected in bovine embryo conditioned medium.
Box plots for biochemical components detected in SOF medium conditioned by bovine embryos with more than eight cells cultured for 3 days (day 5 – day 8 of *in vitro* culture). a) mannitol/sorbitol, b) fructose and c) pyruvate. The upper whiskers represent the maximum, and the lower whiskers the minimum values. The plus-signs indicate the mean values, while the median values are represented by a black line within the boxes. Boxes with different letters differ significantly. Light blue – conditioned glucose-free medium; blue – conditioned 3 mM glucose supplemented medium.

Discussion

Metabolic stress induced by supraphysiological glucose concentrations has previously been reported to alter gene expression in preimplantation embryos [4, 15]. Furthermore, high maternal blood glucose concentrations are thought to compromise embryo development and predispose to gestational complications. In this study, we investigated changes in the metabolomic profile of bovine blastocysts resulting after culture in glucose-supplemented fertilization and embryo culture media in comparison to blastocysts produced in control media.

The glucose concentrations reported for bovine oviductal fluid vary between 0.05 and 4.5 mM and are lower than those detected in blood (between 5.8 and 7.7 mM) [31, 35–37]. In this respect, while it is not too surprising that a glucose concentration of 10 mM appears to be lethal to bovine embryos during *in vitro* culture [15], it is surprising that concentrations of 4.5 mM [38] or 5 mM [15] compromised embryo development, even when elevated glucose was only present during part of the embryo culture period and especially when the glucose was present early in the culture period. In the current study, elevated glucose concentrations were present throughout fertilization and embryo culture up to the blastocyst stage. Although the percentage of blastocysts that developed from oocytes in the presence of 3 mM glucose was reduced, the blastocysts formed appeared otherwise grossly normal and were used for metabolomic analysis.

Early embryos, in the first few days after fertilization, have been reported to primarily utilize pyruvate as an energy substrate [39, 40]; it is only after compaction at the morula stage that glucose becomes an important substrate, via glycolysis, for energy production and lipid biosynthesis. In addition, glucose can enter the pentose phosphate pathway and be used for nucleotide synthesis, NADPH production and regulation of the intracellular redox status [41]. The increased levels of 3-phosphoglycerate and phosphoenolpyruvate in embryos exposed to higher concentrations of glucose indicate increased glycolysis under these conditions. Additionally, the 25-fold increase in sedoheptulose indicates that glucose is also channelled into the pentose phosphate pathway. Glucose-exposed blastocysts also exhibited a 310-fold increase in mannitol/sorbitol concentrations. In this respect, the polyol pathway converts glucose into sorbitol, which is further converted into fructose; increased sorbitol concentrations therefore indicate increased activation of the polyol pathway to generate energy. The increased concentrations of mannitol/sorbitol in

the blastocyst conditioned medium were less expected, because of the poor diffusion of sorbitol through the plasma membrane. On the other hand, sorbitol accumulation in cells is a common feature of diabetes, and leads to accumulation of reactive oxygen species (ROS). The release of sorbitol into the medium might counteract this ROS accumulation in the blastocysts.

Increased N-acetylglucosamine 1-phosphate and UDP-N-acetylglucosamine concentrations suggest engagement of the hexosamine pathway in blastocysts encountering elevated glucose concentrations. In this respect, it has been reported that protein glycosylation via the hexosamine pathway is the underlying mechanism for the embryotoxic effect of excess glucose [42]. It has also been suggested that activation of the hexosamine pathway leads to increased TGFβ-1 expression [43, 44]. However, the transcriptome of glucose-exposed bovine embryos did not show a change in *TGFB1* mRNA expression, although Ingenuity Pathway Analysis did indicate enhanced TGF-β signalling [15].

Hyperglycemic conditions lead to the production of reactive oxygen species, which can stimulate the polyol pathway by inhibiting glyceraldehyde-3-phosphate dehydrogenase activity [44]. It has therefore been suggested that hyperglycemic conditions can trigger the Warburg effect in bovine embryos, in particular anaerobic glycolysis and lactate production [15]. Although pyruvate oxidation via the tricarboxylic acid cycle seemed to be reduced, which is a characteristic of the Warburg effect, we did not observe an increase in intra- or extracellular lactate production by embryos cultured in 3 mM glucose.

Blastocysts stimulated with glucose showed a reduction in oxidized glutathione concentrations, which is also reported to occur under diabetic conditions [45]. Glutathione is a tripeptide synthetized from glycine, cysteine and glutamate. Two of the amino acids, glycine and cysteine were decreased in glucose treated blastocysts, which hints at decreased glutathione synthesis. Patients with uncontrolled type 2 diabetes also showed a decrease in concentrations of the amino acids glycine and cysteine [46]; the lowered levels of glutathione might be a reason for higher levels of oxidative stress during diabetes.

Despite the apparent increase in embryo glycolysis, reduced concentrations of Kreb's cycle components indicate reduced activity of this important energy producing pathway under hyperglycemic conditions (Figure 4). It is therefore possible that high glucose concentrations result in defective mitochondrial oxidative phosphorylation. In any case, reduced tricarboxylic acid cycle activity

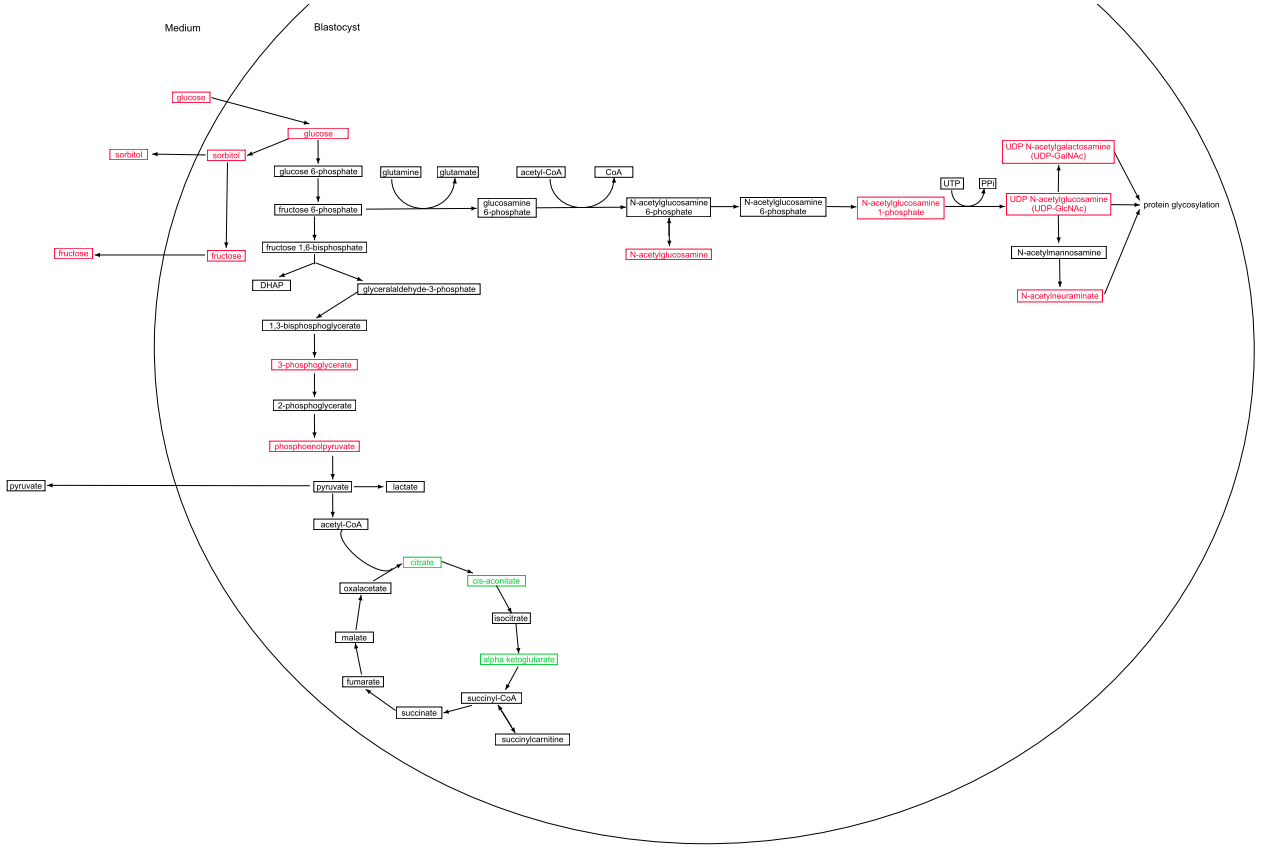

Figure 4: Schematic diagram of biochemicals in bovine blastocysts affected by glucose exposure. Biochemical components of glycolysis, the tricarboxylic acid cycle, and the hexosamine pathway altered when bovine blastocysts were cultured in the presence of 3 mM (compared of 0 mM) glucose. Metabolites shown in green decreased and those in red increased in blastocysts exposed to 3 mM glucose, or in medium conditioned by these blastocysts, compared to control conditions (without glucose).

indicates inefficient energy production under hyperglycemic conditions. As shown in rats [47] and mice [48], citrate and isocitrate are decreased under diabetic conditions. Similarly, the glucose-treated blastocysts in the current study showed a decrease in citrate and, while isocitrate was not detected in the current study, a decrease in isocitrate is possible, since aconitate and α-ketoglutarate were decreased. The decrease in the components in the chain from citrate to α-ketoglutarate might be explained by inhibition of the enzyme aconitase by reactive oxygen species [49, 50]; the unchanged levels of the tricarboxylic acid cycle metabolites, malate and fumarate, might be explained by maintenance of the reaction from α-ketoglutarate to succinyl-CoA by glutamate [50].

In short, most of the changes detected in glucose-stimulated blastocysts were consistent with altered carbohydrate metabolism, and it appears that these blastocysts use the pentose phosphate pathway preferentially to deal with the elevated glucose concentrations in their local environment.

In mouse preimplantation embryos, epigenetic reprogramming, and in particular DNA methylation and histone modifications, take place at the blastocyst stage [51]. Moreover, the nutritional environment to which the early embryo is exposed is able to alter the extent and position of DNA methylations, and can thereby affect the risk of the resulting offspring developing metabolic diseases [52]. In the current study, we expected glucose exposure to lead to altered concentrations of S-adenosylmethionine (SAM), a substrate for methyl-transferases. However, while SAM was detected in cumulus cells (data not shown) it was not detected in blastocysts, irrespective of the glucose content of the culture medium. On the other hand, since entire blastocysts were examined, and differences in DNA methylation are known to exist between trophectoderm and inner cell mass cells, changes in SAM concentrations may have been obscured by differences between the two cell types.

In summary, in this study we describe the effect on metabolic pathways of exposing early bovine embryos to high concentrations of glucose, as would be expected to occur in pregnant diabetic patients. It should be noted that, during diabetes, other tissues/cells including those of the oviduct and uterus will also be exposed to high glucose concentrations which may alter their behaviour and secretory activity accordingly and thereby modify effects seen at the level of the embryo.

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Supplementary Information

Can be found here:
https://drive.google.com/drive/folders/1QeNth8HDYOgu1hM_Fwupsrdy50tdGKlk?usp=sharing


6

Summarizing Discussion

Since the birth of the first IVF baby in 1978 [1], the use of artificial reproductive techniques (ART), including conventional IVF and ICSI, to help couples that have difficulty in conceiving, has increased steadily. Numbers from the European Society for Human Reproduction (ESHRE) indicate that in Europe the numbers of IVF/ICSI cycles is still increasing [2] (Figure 1). The main reasons for the increased use of IVF are thought to include changes in lifestyle; the increased average age of first time mothers and increasing levels of obesity in the western world are factors that are known to reduce fertility. In addition to 'regular' IVF, new technologies are being used to help 'preserve' fertility. Nowadays for example, women can choose to freeze oocytes when they are still at a 'good' reproductive age, preferably between 20 and 30 years old, for later use, a process referred to as 'social freezing'. Oocyte freezing was originally developed for female cancer patients, to secure healthy oocytes before undergoing potentially damaging chemo- or radiotherapy. Social freezing is, however, controversial and has been heavily debated, particularly after Facebook and Apple offered to cover the costs of oocyte freezing for their female employees in 2014, ostensibly to help them achieve their career goals before considering maternity leave. Even more ethically challenging is

the potential for generating germ cells from adult somatic cells after nuclear reprogramming [3]. In mice, live progeny have been obtained from germ cells produced by directed differentiation of induced pluripotent stem cells [4].

Although IVF was developed 40 years ago, the overall efficiency is still below 50%, which means that many women have to undergo several cycles to become pregnant and, in a significant percentage of women, it will never be successful [5]. In addition, since many oocytes are fertilized, whereas only one embryo is usually transferred into the uterus, many embryos are leftover. To increase IVF efficiency and prognosis, it would be useful to be able to predict the developmental competence of oocytes. Cumulus cell properties could possibly help to identify enclosed oocytes with the competence to develop into a healthy embryo likely to yield a healthy child.

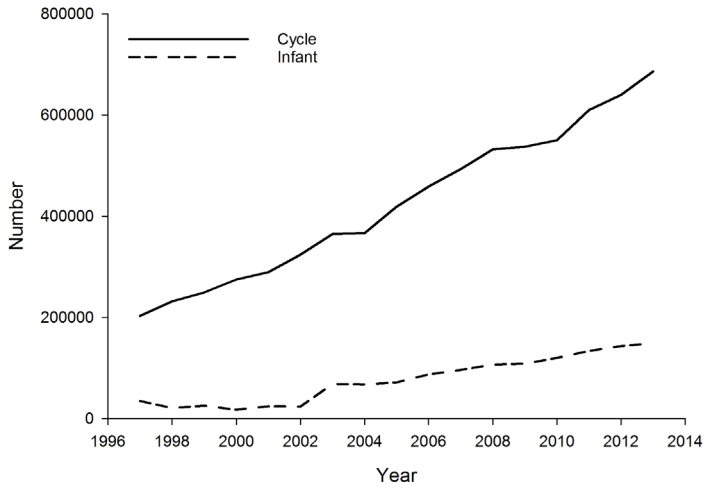

Figure 1: Numbers of IVF/ICSI cycles and infants in Europe.
IVF and ICSI cycles were registered by the ESHRE [2], the solid line represents the cycles, the dashed line the number of infants born.

microRNAs and mRNAs in cumulus cells

During maturation, the cumulus cells secrete a range molecules to build up an extracellular matrix, this serves not only to protect the oocyte during transport into and through the oviduct [6], but also plays an important role in normal oocyte maturation. For instance, the cumulus cells provide energy substrates required during maturation, and convert glucose into metabolites that can

be used by the oocyte. In addition, the cumulus cells express microRNAs (miRNAs), which may help to post-transcriptionally regulate mRNA expression in the oocyte and early embryo up to the time of embryonic genome activation. Cumulus cell mRNAs are also important during maturation and in supporting the oocyte's attainment of developmental competence, and levels of cumulus cell mRNAs are also likely to be regulated by miRNAs.

Until now the function of miRNAs during oocyte maturation and early embryonic development has not been investigated extensively. In mice, it has been suggested that small noncoding RNAs are essential for oocyte maturation and embryo development [7, 8], although other studies showed that genetic deletion of *Dgcr8* did not interfere with formation of normal oocytes, which yielded apparently normal offspring after fertilization. Unlike DICER which is also involved in the maturation of other small RNAs, DGCR8 is exclusively involved in miRNA maturation and it was therefore concluded that, at least in the mouse, miRNAs are not essential for oogenesis and embryo development [9–11]. In other species, such as the zebrafish, cow and man however, miRNAs have been shown to regulate the activity of mRNAs important for follicle growth, oocyte maturation and embryo development [12–15].

In **chapter 2,** sequencing of miRNAs in bovine cumulus cells enclosing oocytes that either developed into a blastocyst or failed to cleave after fertilization, demonstrated that the overall miRNA expression pattern did not differ significantly between the two groups. We concluded that the level of expression of single miRNA species cannot be used to predict the developmental competence of the enclosed oocyte. Possibly, pooling of cumulus cell samples masked differences between individual cumulus complexes, while there may also be multiple routes to mRNA regulation and thereby redundancy among individual miRNAs. Indeed, the expression levels of selected miRNAs, as examined by qRT-PCR, showed large variations even between individual samples belonging to the same group (blastocyst versus non-cleaved). Since we used slaughterhouse material, an effect of breed or age on miRNA expression can also not be excluded. MiRNA sequencing of cumulus cells from individual COCs might help to identify miRNAs important to oocyte developmental competence, and ratios of different miRNAs in cleaved versus non-cleaved groups might help to identify miRNAs that are important during maturation. However, it is also possible that miRNA expression levels in different cumulus cells from the same COC differ, depending for example on the distance to the enclosed oocyte.

Of all miRNAs identified in bovine cumulus cells, miRNA-21 showed the highest levels of expression. In **chapter 3,** we found that miRNA-21 expression changes over time during maturation, but did not differ between inner and outer cumulus cells. A difference in expression between inner and outer cumulus cells might have been expected, because miRNA-21 expression has been reported to be induced through TGFβ and BMP signalling [16]. GDF-9 and BMP-15 are TGFβ like oocyte-secreted factors, and a gradient in BMP-15 from the inner to the outer cumulus cells has been described. A similar gradient was also found using an apoptotic cell assay on cumulus cells, i.e. outer cumulus cells were more likely to be apoptotic than inner cumulus cells [17]. An anti-apoptotic effect was therefore attributed to miR-21 [18]; however, our data do not support this theory given the similar level of expression of miR-21 in inner and outer cumulus cells.

An *in vitro* maturation experiment showed that inhibiting miR-21 compromised oocyte maturation and reduced oocyte developmental competence [19]. Gene regulation was also apparently influenced by miR-21. In human cumulus cells, miR-21 and miR-29a were found to jointly target DNA methyltransferases (DNMTs) [20, 21], which are upregulated in MII oocytes. DNMTs are enzymes that regulate the methylation of DNA, and the DNMTs are therefore important gene regulating elements. MiRNAs can traffic from the cumulus cells to the oocyte [15] and, if transport of miR-21 or miR-29a was altered, abnormal DNMT control of methylation could result, possibly influencing reproductive potential or even health of the offspring [22]. In **chapter 3,** we investigated how the expression profiles of selected genes with a possible link to oocyte developmental competence changed over the time-course of maturation, and whether inner and outer cumulus cells differed in their expression profiles. With the exception of *HAS2*, the expression of all tested genes changed during maturation. At 8h of maturation, *HAS2* expression was higher in outer than inner cumulus cells. Although the cumulus cells are derived from the same granulosa cell precursors, these data suggest that inner cumulus cells behave differently to outer cells and that they may be distinguished as two functionally separate cell populations. Experiments to analyse protein expression, combined with individual culture of the different cell subsets could provide more information.

After *in silico* prediction of mRNAs potentially targeted by miRNAs, reporter assay systems can be used to determine whether a mRNA species is indeed targeted by a specific miRNA. Here, we used a luciferase assay to test whether

CD44 and *SLC38A2* were targets for miR-25 and whether miR-155 targeted *SMAD2* or *WEE1*. Contrary to expectations, we were not able to prove targeting of any of the tested candidate mRNAs by the miRNAs included in our experimental set-up. This confirms that the *in silico* target prediction tools identify false positive targets, but also emphasizes that selecting the most likely target of a miRNA is not straightforward. A substantial disadvantage of the reporter assay system that we used is that it can only examine the interaction between a single mRNA-miRNA pair. Methods to examine a collection of potential targets of a given miRNA include immunoprecipitation techniques in which the miRNA-mRNA pair is precipitated with antibodies against RISC components such as AGO, followed by high-throughput sequencing of RNA (HITS-CLIP) [23], and biotin tagging of miRNA [24]. Alternatively, miRNAs that target a specific mRNA can be identified by an affinity purification method [25]. It would be interesting to identify miRNAs able to target genes important for cumulus expansion, like *HAS2* or *CD44*, to better understand how and to what extent miRNAs regulate mRNAs in cumulus cells.

Metabolomics

To date, no reliable markers for the prospective assessment of the developmental competence of an oocyte or the quality of a resulting blastocyst have been identified; therefore, it is common to assess oocyte and blastocyst quality by microscopy. Microscopic evaluation is rather subjective and depends on the experience of the evaluator. Another difficulty is that the morphology does not always reliably reflect the 'true' quality of the oocyte or embryo, for example because genetic or epigenetic defects are not detected; nevertheless, microscopy is quick and inexpensive [26]. The analysis of metabolites released into 'conditioned' medium by oocytes during *in vitro* maturation or by developing embryo's during *in vitro* production might be useful for determining the quality of oocytes or embryos. Metabolic profiling has, for instance, been successfully used to detect trisomy 21 in embryos [27]. Theoretically, metabolic profiling of conditioned medium or cumulus cells may yield information with the power to predict the developmental competence of an oocyte, but this will require thorough understanding of metabolic processes in cumulus cells and how they promote, support or compromise oocyte developmental competence. This knowledge would enable supplementation of media with factors likely to enhance the developmental competence of an oocyte or embryo. **Chapter 4** shows that COCs help create an environment beneficial for oocyte maturation. If oocytes were matured for 8h within their COCs, denuded and returned to their own conditioned maturation medium to

complete the maturation process, the likelihood of blastocyst formation after fertilization was higher than when oocytes were transferred to fresh maturation medium after denudation. On the basis of these findings, a metabolic profile of the cumulus cells and conditioned maturation medium was generated at different time points during maturation, namely the GV-stage, 8h after the onset of maturation, and 23h after the onset of maturation (**chapter 4**). Of the components found to change significantly in concentration during maturation, the functions of carnitine and creatine were explored further. They were chosen because carnitine increased in the medium within the first 8h of maturation, and metabolites of carnitine decreased in the cumulus cells during this time, suggesting further metabolism; the carnitine pathway is important for the transport of fatty acids into the mitochondrion. Creatine was chosen because of the 427-fold increase in the medium within 8h of maturation; creatine is important for ATP provision to the oocyte. Unlike carnitine, creatine supplementation did not affect the success of blastocyst formation. It has previously been demonstrated that cumulus cells can protect the oocyte they enclose from fatty acids, by sequestering free fatty acids into lipid droplets [28]. Other studies have reported that the lipid content of the cumulus cells is an indicator of oocyte quality [29, 30]. Together with the inability of the oocyte to produce cholesterol, a task instead performed by the cumulus cells [31], this suggests that lipid accumulation and metabolism by cumulus cells influences developmental competence of the oocyte.

Another important energy substrate for the developing oocyte/embryo is glucose. However, at high concentrations glucose can have detrimental effects. Concentrations of >10 mM glucose in *in vitro* culture medium is lethal to bovine blastocysts [32]. In our studies, even the presence of 3 mM glucose during *in vitro* fertilization and embryo culture significantly decreased the likelihood of blastocyst development compared to control conditions, i.e. without glucose (**chapter 5**). Chronic hyperglycaemia, i.e. high fasting glucose concentrations in blood plasma, is a defining characteristic of diabetes. Although it has been reported that the glucose concentration in oviductal fluid is lower than that in blood [33–36], when the blood glucose concentration increases so does that in the reproductive tract [37]. The metabolic profile of blastocysts exposed to high glucose concentrations during development *in vitro* showed an upregulation of components and pathways typical of diabetes, such as glycolysis and the hexosamine pathway, accompanied by a decrease in the tricarboxylic acid cycle. It has also been shown that even transient exposure to elevated glucose concentrations compromises

embryo development [32, 38]. In mice, it has been reported that single cell zygotes transferred from a diabetic mother to a non-diabetic mouse still suffer diabetes-induced consequences, like congenital malformations of the resulting offspring [39], and that maternal diabetes affects the transcriptome of the embryo [40]. Maternal diet could also influence the epigenome of the embryo, for instance by altering the pattern of histone acetylation [41]. One long term effect on offspring exposed to prenatal diabetes, is a higher risk of diabetes or cardiovascular diseases [42, 43]. Another effect of maternal hyperglycaemia is a build-up of reactive oxygen species in the embryo, which leads to increased apoptosis [44]. Accumulation of sorbitol in the cells is partially responsible for the increase in reactive oxygen species, and metabolic profiling in our study showed an increased sorbitol level in glucose-exposed blastocysts. Indeed, our *in vitro* culture model revealed changes typical of diabetes, suggesting that it could be an appropriate model to investigate pathways activated in embryos exposed to a hyperglycaemic environment. Of course, it does not mimic the whole *in vivo* situation because the cells of the maternal reproductive tract also interact with the embryo. However, the bovine model has advantages over more commonly used rodent models. For example, mouse IVF is more vulnerable to nutrient changes; indeed, if mouse zygotes are cultured without nutrients, they degenerate within 10h [45, 46], whereas bovine zygotes are able to pass through the first cleavage divisions without supplementary nutrients [45]. In addition, while mouse zygotes have been shown to tolerate glucose concentrations up to 30 mM, bovine zygotes will not survive glucose concentrations above 10 mM [32]. The preimplantation embryos of man and cow also have similar biochemical processes, as well as intrinsic paternal and maternal regulatory processes [47]. Culturing bovine embryos in a diabetes-mimicking environment could help to understand the effects on specific pathways, help predict resulting aberrations and malformations better and to a greater extent, and thereby partially replace *in vivo* animal models with their associated ethical restrictions.

Conclusion

Although all cumulus cells derive from the same basic cell type, they seem to have different tasks depending on their exact location with respect to the oocyte. The expression of miRNAs and mRNAs in cumulus cells also change during the course of oocyte maturation, and the inner cumulus cells are clearly different to the outer cumulus cells. In the future, cumulus cells should not be considered as a uniform group of cells. It should be taken into account that miRNA and mRNA expression profiles change over the time course of

maturation, and differ in the different parts of the cumulus complex. Since the inner cumulus cells are in closer contact with the oocyte, it is possible that they would yield more useful information over the developmental competence of the oocyte. The role of miRNAs in cumulus cells is not yet clear, but they do seem to be important for successful maturation, even if they do not yet seem to be useful as markers to predict the developmental competence of the oocyte. A closer look at gene expression and regulation thereof might also help identify metabolites important during maturation and that could be used to enhance the developmental competence of an oocyte. Within the first 8h of maturation *in vitro*, the COCs create a microenvironment through their secretions that supports the further acquisition of developmental competence. Which particular factors play these supportive roles needs to be examined further. Nevertheless, it appears that carnitine, via its role in fatty acid metabolism, has a positive effect on oocyte maturation. However, other metabolites or pathways that were not tested further as part of this thesis may also have important roles to play, for example, glycogen metabolism or nucleotide sugar metabolism.

The timing of cumulus cell removal and the location of the cumulus cells could be the starting points for future investigations into the roles of cumulus cells in, or their use as markers for, oocyte developmental competence.

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Summary

Assisted reproductive technology (ART) is commonly used to help subfertile couples to achieve pregnancy, and in economically valuable animals, like cattle, to efficiently generate progeny from a valuable female with the use of different males. In addition ART has been used to try to preserve endangered species. Although assisted reproductive technology, like *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) has been increasingly used during the last decades, the efficiency is far from optimal. Although the adult ovary contains thousands of oocytes, not every oocyte is able to develop into an embryo after fertilization. Exactly what defines a developmentally competent oocyte is not known, however.

Developmental competence is defined as the possibility of the oocyte to develop into a viable foetus after fertilization. Knowledge about the competence of oocytes would be beneficial to avoid surplus embryos resulting from the fertilization of several oocytes using assisted reproductive technology. Therefore, it would be useful to better understand the processes during oocyte maturation, to identify what defines the developmental competence of an oocyte.

An important factor during oocyte maturation is the complex of cumulus cells, somatic cells surrounding the oocyte. Cumulus cells are important for different processes during maturation, for instance the conversion of glucose to pyruvate, that is used by the oocyte as energy substrate. Cumulus cells are connected with each other and with the oocyte, enabling a bidirectional communication. This close relation between cumulus cells and oocytes could possibly be used to predict the developmental competence of an oocyte by analyses of the cumulus cells.

In chapter 2 it was tested whether the microRNA (miRNA) expression profile of cumulus cells can predict the developmental competence of the enclosed oocyte. MiRNAs are small, non-coding, RNAs, which are able to regulate mRNAs levels posttranscriptionally. It is possible that one microRNA species regulates ~100 different mRNA sequences. Since miRNAs can potentially regulate a range of mRNAs, the expression of miRNAs in the cumulus cells was investigated. Cumulus complex biopsies were taken from individual cumulus-oocyte complexes after maturation, and the enclosed oocytes and embryos were individually fertilized and cultured to distinguish cumulus complexes from developmentally competent versus those from incompetent oocytes. Small RNA sequencing was used to identify miRNA profiles. The

result of the sequencing showed no differences in miRNA expression between the two tested groups. Further expression analysis by qRT-PCR showed a high variability of miRNA expression within individual cumulus complexes.

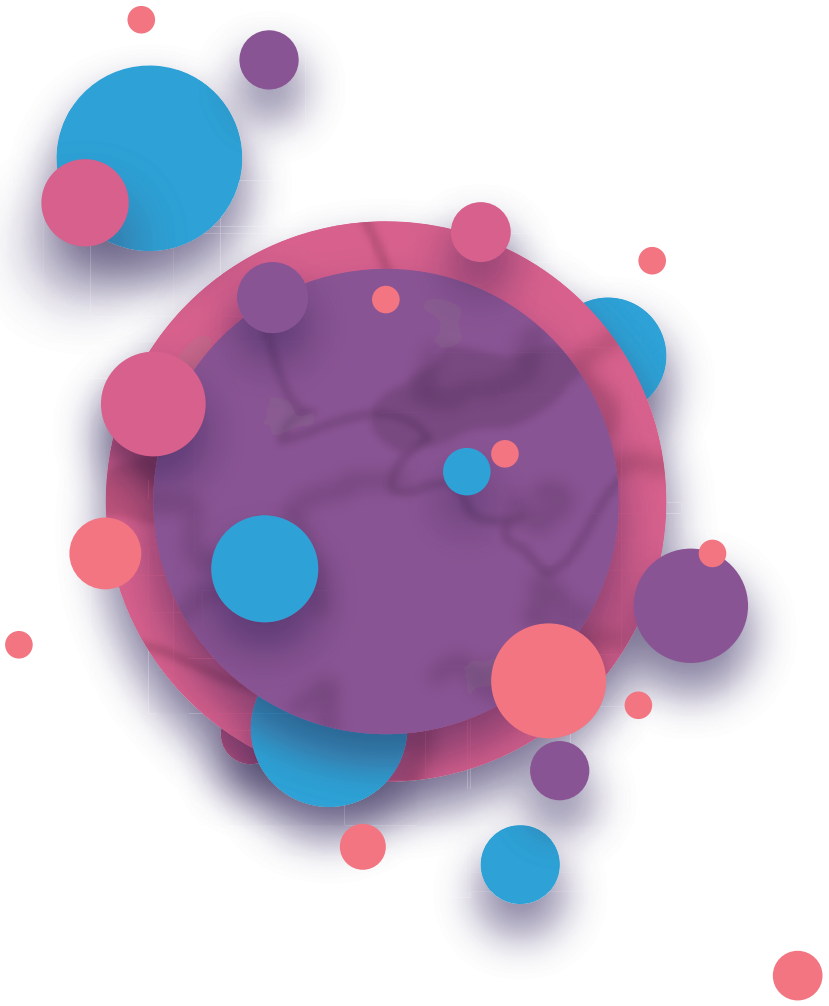
On basis of the sequencing results, in chapter 3 the expression profiles of several miRNAs were further examined, in particular their expression during the course of maturation spatial differences within the complex inner and outer cumulus cells. Among the tested miRNAs were miR-21 and miR-155 which showed an increased expression over the course of maturation and miR-25 and miR-125 which showed an increased expression in inner cumulus cells compared to the outer cumulus cells. Additionally, mRNA expression of predicted targets, using *in silico* methods, of these miRNAs were investigated. Among this mRNAs were *CD44* and *SLC38A2* as targets for miR-25 and *SMAD2* and *WEE1* as targets of miR-155. To verify if the miRNAs can regulate the mRNAs a reporter gene assay was performed, which did not show a regulation between the mRNA-miRNA pairs.

To show the importance of cumulus cells during maturation cumulus cells were removed 8h after onset of maturation, after which the denuded oocytes were either placed back in their own conditioned medium or in completely fresh maturation medium (chapter 4). After fertilization, a lower percentage of cleaved zygotes were found if the oocytes were cultured in fresh maturation medium compared to oocytes cultured in their conditioned medium. Also, the percentages of blastocysts were lower if the denuded oocytes were placed into fresh maturation medium, compared to conditioned medium. On basis of these results, it is concluded that cumulus cells secrete molecules, which support the developmental competence of the oocyte. To identify these biochemicals a metabolomic analysis was done from the cumulus cells and the maturation medium. Two of the identified biochemicals were tested further, carnitine and creatine. Whereas carnitine showed a beneficial effect on cleavage and blastocyst formation, this effect was not obtained with creatine supplementation to the maturation medium.

Metabolic diseases like diabetes influence the oocyte quality. It is known that women suffering from diabetes have a higher risk for abortion and congenital malformations. To understand the metabolic processes, which occur in early embryos exposed to elevated glucose concentrations, a metabolomic analysis on day 8 bovine blastocysts was performed (chapter 5). During fertilization and embryo culture 3 mM glucose was present in the medium, to simulate

elevated glucose levels occurring in the reproductive environment of diabetic women. The comparison of unstimulated and stimulated blastocysts showed that the glycolysis and the activity of the hexosamine pathway were increased if the blastocysts were cultured under elevated glucose concentrations. On the other hand, components of the tricarboxylic acid cycle were reduced, suggesting an inefficient energy production from pyruvate. Taken together, the glucose stimulated blastocysts showed typical changes characteristic for a diabetic environment. This system can be further used to investigate and better understand the processes in embryos exposed to elevated glucose concentrations.

The results of this thesis showed that, although the cumulus cell complex appears to be a uniform cell mass, differences exist between the individual cumulus cells in terms of mRNA and miRNA expression throughout time and dependent on the location of the cumulus cells. The cumulus-oocyte complex is also responsible to create a microenvironment which is beneficial during the maturation process and the developmental competence of the enclosed oocyte.



Nederlandse samenvatting

Kunstmatige voortplantingstechnieken worden vaak gebruikt in humane klinieken bij onvervulde kinderwensen, en bij economisch waardevolle dieren, zoals vee, om meer nakomelingen van een vrouwelijk dier te krijgen met het gebruik van verschillende mannelijke dieren als vader. Hoewel kunstmatige voortplantingsmethoden zoals in vitro fertilisatie (IVF) of intracytoplasmatische sperma-injectie (ICSI) de laatste decennia al worden gebruikt, is de efficiëntie verre van optimaal. Een reden hiervoor is dat niet iedere eicel zich na bevruchting kan ontwikkelen tot een embryo, maar tot nu toe is het niet precies bekend wat bepaalt of een eicel ontwikkelingscompetent is. De ontwikkelingscompetentie wordt gedefinieerd als de mogelijkheid dat de eicel zich na bevruchting ontwikkelt tot een levensvatbaar nageslacht. Deze kennis over ontwikkelingscompetentie zou ook gebruikt kunnen worden om de productie van overtollige embryo‘s bij kunstmatige voortplantingsmethoden te vermijden, omdat nu alle bruikbare eicellen worden bevrucht, ook die eicellen die geen levensvatbare foetus zullen vormen. Om bovenstaande redenen is het nuttig om de processen tijdens de eicelrijping beter te begrijpen en om te bepalen wat belangrijk is voor de ontwikkelingscompetentie van een eicel.

Cumuluscellen, somatische cellen die de eicel omgeven, vervullen een belangrijke rol tijdens de eicelrijping. Cumuluscellen zijn belangrijk voor verschillende processen tijdens de eicelrijping, ze zetten bijvoorbeeld glucose om in pyruvaat, het energiesubstraat waar de eicel voorkeur aan geeft. De cumuluscellen zijn met elkaar verbonden door zogenaamde gap junctions en de binnenste laag van deze cellen is verbonden met de eicel via transzonale cytoplasmatische uitlopers. Door deze nauwe relatie vindt een bidirectionele communicatie plaats. Deze nauwe relatie tussen cumuluscellen en de eicel maakt de cumuluscellen ook tot een interessant onderzoeksobject voor kunstmatige voortplantingstechnieken, om reeds vóór de fertilisatie de ontwikkelingscompetentie van een eicel te voorspellen.

In hoofdstuk 2 werd onderzocht of de microRNA (miRNA) expressie in de cumuluscellen de ontwikkelingscompetentie van de ingesloten eicel kan voorspellen. MiRNA‘s zijn kleine, niet-coderende RNA‘s, die in staat zijn mRNA‘s posttranscriptioneel te reguleren. Het is mogelijk dat één microRNA de functie van ~ 100 verschillende mRNA‘s reguleert. Omdat de cumuluscellen de mogelijkheid hebben om een reeks mRNA‘s te reguleren, werd in dit hoofdstuk de expressie van miRNA‘s in de cumuluscellen onderzocht met behulp van miRNA *sequencing*. Na rijping werd van elk cumulus-eicel

complex afzonderlijk cumuluscelbiopten genomen, ook werden bevruchting en in vitro embryokweek afzonderlijk uitgevoerd om retrospectief te bepalen of de cumuluscellen een eicel omsloten die in staat was om zich tot blastocyst te ontwikkelen of ongedeeld bleef na bevruchting. Er werden geen duidelijke verschillen in miRNA-expressie tussen de twee geteste groepen gevonden. Verdere expressie-analyse door qRT-PCR vertoonde een hoge variabiliteit van miRNA-expressie binnen één groep.

Op basis van de *sequencing* resultaten werden stabiele uitgedrukte miRNA's getest in hoofdstuk 3, er werd getest hoe de expressieniveau's van de miRNA's tijdens de rijping veranderen en of er een verschil in expressie tussen cumuluscellen dichter bij de eicel (cellen van de binnenste cumulus) of cellen die verder verwijderd zijn van de eicel (buitenste cumuluscellen). Onder de geteste miRNA's bevonden zich miR-21 en miR-155 die een toenemende expressie vertoonden in de loop van de rijping en miR-25 en miR-125 die een verhoogde expressie vertoonden in cellen van de binnenste cumulus vergeleken met de buitenste cumuluscellen. Bovendien werd mRNA-expressie van potentiele target mRNA's met behulp van *in silico*-methoden van deze miRNA's onderzocht. Onder deze mRNA's waren *CD44* en *SLC38A2* als doelen voor miR-25 en *SMAD2* en *WEE1* als doelen van miR-155. Om te verifiëren of de miRNA's de mRNA's kunnen reguleren, werd een reportergenassay uitgevoerd, die geen regulatie tussen de mRNA-miRNA-paren vertoonde.

Om het belang van cumuluscellen tijdens eicelrijping te laten zien 8 uur na het begin van de rijping werden de kale eicellen ofwel terug in hun eigen geconditioneerde medium ofwel in volledig vers maturatiemedium geplaatst (hoofdstuk 4). Na de bevruchting werd een lager percentage van gekliefde eicellen gevonden als de eicellen in vers rijpingsmedium werden geplaatst in vergelijking met eicellen die in hun geconditioneerde medium werden geplaatst. Ook was het percentage blastocysten lager indien eicellen zonder cumuluscellen in vers rijpingsmedium werden geplaatst, vergeleken met geconditioneerd medium. Er is geconcludeerd dat het cumuluscellen factoren uitscheiden die de ontwikkelingscompetentie van de eicel ondersteunen. Om deze moleculen te identificeren werd een metabolische analyse uitgevoerd van de cumuluscellen en het rijpingsmedium. Twee van de geïdentificeerde biochemicaliën werden verder getest, carnitine en creatine. Terwijl carnitine een gunstig effect op de klieving en blastocystvorming vertoonde, werd dit effect niet verkregen met creatine suppletie aan het rijpingsmedium.

Het is bekend dat vrouwen die lijden aan diabetes een hoger risico hebben op abortus of aangeboren misvormingen en waarschijnlijk beïnvloeden stofwisselingsziekten zoals diabetes de kwaliteit van de eicel. Om de metabole processen die voorkomen in vroege embryo's als ze worden blootgesteld aan verhoogde glucoseconcentraties beter te begrijpen, werd een metabolomische analyse op dag 8 blastocysten uitgevoerd (hoofdstuk 5). Tijdens de embryokweek werd 3 mM glucose aan het medium toegevoegd om verhoogde glucosewaarden te simuleren die optreden in de reproductieve omgeving van vrouwen met diabetes. De glycolyse en de activiteit van de hexosamine-route was toegenomen bij kweek van blastocysten onder verhoogde glucoseconcentraties. Aan de andere kant waren componenten van de tricarbonzuurcyclus verminderd, dit duidt op een inefficiënte energieproductie uit pyruvaat. Samengevat vertoonden de door glucose gestimuleerde blastocysten typische veranderingen, die kenmerkend zijn voor een diabetische omgeving. Dit systeem kan verder worden gebruikt om de processen in embryo's die worden blootgesteld aan verhoogde glucoseconcentraties beter te onderzoeken en te begrijpen.

De resultaten van dit proefschrift laten zien dat, ook als cumuluscellen een uniforme celmassa lijken te zijn, ze een verschillende en dynamisch mRNA en miRNA expressie , ook afhankelijk van de positie in het cumuluscomplex. Het cumulus-eicel complex is ook verantwoordelijk voor het creëren van een micro-omgeving die gunstig is gedurende het rijpingsproces en de ontwikkelingscompetentie van de ingesloten eicel.

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Curriculum Vitae

Karen Uhde was born on July 12th in Salzwedel, Germany. In 2007, she finished her secondary education at the "Geschwister Scholl Gymnasium" in Gardelegen. Thereafter, she studied Biochemistry at the Martin Luther University Halle-Wittenberg. In 2011 she received her Bachelor of Science degree (B.Sc.) in Biochemistry. During her bachelor thesis, she worked with cloning and expression of bacterial glutaminyl cyclase. This thesis was done at Probidrug AG Halle/Saale. In 2013 she received her Master of Science degree (M.Sc.) in Biochemistry. She did her Master thesis at the Department of Anatomy and Cell Biology, Martin Luther University Halle-Wittenberg. During her Master thesis she investigated the regulation of forkhead box protein O in rabbit blastocysts. Afterwards she started her PhD at the Department of Farm Animal Health of the Faculty of Veterinary Medicine, Utrecht University. The PhD track was founded by the European Commission, within the Marie Sklodowska-Curie Action, for the EpiHealthNet project (FP7-PEOPLE-2012-ITN), during this time she had the opportunity to collaborate with other partners of the EpiHealthNet project, the Martin Luther University Halle-Wittenberg and Metabolon Inc., Durham, North Carolina, USA. The results of her research are described in this thesis.

List of Publications

K. Uhde, H.T.A. van Tol, T.A.E. Stout and B.A.J Roelen; MicroRNA Expression in Bovine Cumulus Cells in Relation to Oocyte Quality. ***Non-Coding RNA*** 2017, ***3***(1), 12; doi:10.3390/ncrna3010012

Awards

International Embryo Transfer Society 42nd Annual Conference 20016, Louisville, Kentucky, (USA) - Peter W. Farin Travel Award
49th Annual Conference of Physiology & Pathology of Reproduction and also 41st Joint Conference in Veterinary and Human Reproductive Medicine, Leipzig (Germany) – runner up poster prize

Oral presentations

K. Uhde, H.T.A. van Tol, T.A.E. Stout and B.A.J Roelen 2016. MicroRNAs in bovine cumulus cells. Annual meeting 'Vereiniging voor fertiliteitsstudie', Utrecht

Poster presentations

K. Uhde, H.T.A. van Tol, T.A.E. Stout and B.A.J Roelen; Cumulus-Oocyte-Complex Secretions from the first 8 hours of *in vitro* maturation affect oocyte developmental competence. *Reproduction, Fertility and Development* 28(2) 163 (2015) http://dx.doi.org/10.1071/RDv28n2Ab66

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