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OMICS for the Identification of Biomarkers for Oocyte Competence, with Special Reference to the Mare as a Prospective Model for Human Reproductive Medicine

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1. Introduction

A crucial component of Assisted Reproductive Technologies (ART) is the assessment of oocyte developmental potential, to allow selection of those oocytes most likely to result in fertilization and pregnancy. Currently, oocyte quality assessment is largely based on the morphological appearance of the cumulus-oocyte complex, however the accuracy of morphological methods, as predictive of oocyte competence, is still suboptimal. Therefore, the development of objective, accurate, fast and reliable tests for assessing oocyte developmental potential remains an important aim of human and veterinary reproductive medicine. The process of oocyte meiotic maturation, which is central to the developmental competence of the oocyte, is regulated by numerous genes (Matzuk & Lamb, 2008; Fauser et al., 2011) and protein pathways (Kubiak, 2011) and is accompanied by significant changes within the oocyte at many levels. Better understanding of oocyte meiotic maturation would allow better support of this process to increase the success of reproductive biotechnologies, and thus overcome some forms of infertility. Recently, global assessment strategies, namely OMICS, investigating genomic, transcriptomic, proteomic, lipidomic, and glycomic profiles of oocytes, cumulus or granulosa cells have become increasingly applied to the study of oocyte physiology and pathology. Also being investigated is the oocyte-cumulus metabolome, via measurements of metabolites in biological fluids, such as follicular or tubal fluid, or in culture media. The establishment of these technologies, which are in their initial stages of application to reproductive biology, can require large sample numbers; only animal models can meet this requirement. Because of their wide availability and the body of existing knowledge regarding their biology, oocytes of large animals provide useful models for investigating the relationship between oocyte developmental competence and OMICS biomarkers. This review summarizes recent literature on the application of OMICS strategies to evaluating developmental competence of human oocytes and oocytes of large

animals. Among the available animal models, the mare is uniquely applicable to investigation of oocyte developmental competence. Horses represent the most economically valuable domestic animal, with progeny from specific mares worth hundreds of thousands of euros. Thus, there is obvious practical interest in the use of assisted reproduction in this species. In addition, similarities between equine and human follicle growth and oocyte maturation make the mare a particularly valuable model for topics at the interface between animal breeding and biomedical research, such as age-related and obesity-related oocyte dysfunction and the effects of exposure to environmental toxicants, as well as for fundamental research on factors involved in meiotic maturation. For these reasons, particular attention will be dedicated in this review to recent OMICS results obtained in the equine species and to discussion of the potential application of this animal model in future investigations.

2. OMICS as innovative strategies for evaluating oocyte quality

Systems biology is a new and rapidly developing research area in which, by quantitatively describing the interactions among the components of a cell, a systems-level understanding of a biological response can be achieved. Therefore, it requires high-throughput measurement technologies, that is, technologies that can investigate a large number of biological molecules at once. OMICS technologies -- in which aspects of cellular structure or function, such as proteins or RNA transcripts, are studied in their totality (global assessment strategies) -- are opening wider and wider doors into the understanding of all branches of the biology, physiology, and pathology of living organisms. It is likely that information obtained using OMICS will change our concept of "normal" and "pathological," and will enable the efficient evaluation of the effects of extrinsic factors on the status of living systems. Initial studies on the application of OMICS strategies to the oocyte have appeared in the past decade, starting with genomics and transcriptomics, and progressing to the newer fields of glycomics and metabolomics. As noted above, a major concern in the production of viable and competent embryos in vitro is the evaluation of initial oocyte quality and the support of optimal nuclear and cytoplasmic maturation. OMICS approaches to the oocyte will significantly contribute not only to accurate assessment of oocyte quality, but also to the clarification of the mechanisms involved in cell cycle regulation and cell differentiation, thus contributing to the effective utilization of recovered oocytes. Because meiotic maturation and early embryo development involve regulation of the cell cycle and evolution from differentiated, to pluripotent, back to differentiated cells, data generated from study of these processes may also relate to the establishment of innovative targeted cancer treatments and stem cell-based therapies. To introduce the sequential phases of the meiotic process, changes occurring within the oocyte and some of their fundamental regulating factors are briefly described. Upon the luteinizing hormone (LH) surge, M-phase promoting factor (MPF) and the mitogen-activated protein (MAP) kinase ERK 2 (extracellularly-regulated kinase 2) are activated within the oocyte. The oocyte, which at this time is in prophase of meiosis, has replicated chomosomes contained within a nucleus (termed the germinal vesicle, GV). Activation of MPF and ERK 2 trigger nuclear envelope breakdown and chromatin condensation. The condensed chromosomes are subsequently aligned on the spindle of the first meiotic division, forming the metaphase I plate. At this time MPF levels decrease, while ERK 2 levels remain high. The homologous chromosomes

separate: one set of sister chromatids is discarded as the polar body; the other set, in response to a recrudescence of MPF, lines up on a spindle, forming the second metaphase plate (MII). Fertilization causes inactivation of MPF and ERK 2, and the second meiotic division occurs, separating the sister chromatids. One set of chromatids is discarded, as the second polar body; the other set becomes the female pronucleus. For a comprehensive description of major pathways involved in oocyte M-phase entry, see Kubiak et al., 2011 and Tosti & Boni, 2011. Detailed descriptions of OMICS techniques go beyond the intent of this article; corresponding references are provided in the text. We instead focus on the most significant results obtained using these techniques, the role of large animal models in experimental designs that cannot be performed in humans, and on actual and potential contributions of different animal models to understanding of oocyte biology, with particular interest in the equine species.

2.1 The mare as a model for human oocyte biology

Large animal models allow the establishment of a wide variety of experimental designs that can not be applied in humans for obvious ethical reasons, or due to the limited and highly regulated availability of human biological samples. Among large animals, the mare has many attributes that make her a good model for reproduction in women (Carnevale, 2008). These include a long follicular phase, a long interovulatory interval (22 days), presence of a single dominant follicle, formation a large diameter follicle (~40 mm) with a large volume of follicular fluid -- the same volume:body weight ratio as in women; a relatively long time from LH stimulation to ovulation (36 h for both human and horse) and, like the human oocyte, formation of a markedly dense chromatin mass within the germinal vesicle as the oocyte gains meiotic competence or undergoes atresia (Parfenov et al, 1989; Hinrichs et al., 1993). Although seasonality does not occur in women, the equine characteristic of seasonal reproductive activity provides the potential to examine the influence of applied factors when cyclic hormonal patterns are not occurring (Carnevale, 2008). Horses are the best animal model for studies on age-related infertility. Because mares can be of great value, many mares continue to be bred until they experience subfertility, thus animals with naturally-occuring age-related subfertility are available for study. Horses have a long lifespan, thus age-related subfertility occurs at an age (~20-25 years) much closer to that observed in women than is seen in other animal models. In addition, horses, unlike other large domestic species, are selected for attributes other than fertility, such as conformation, athletic prowess, or behavior. Individuals showing subfertility may be worked with intensively to try to obtain foals, thus, they provide an excellent naturally-occurring model for many intrinsic causes of subfertility. Horses have similar metabolic responses to nutrient intake to that in humans, and are used for a wide variety of athletic purposes, thus they can serve to model important physiological or pathological situations affecting reproduction in humans (such as stress, life-style, sports activity, obesity or metabolic syndrome) as well as to examine the effects of external factors such as acute or long-term exposure to drugs or environmental toxicants. In addition to mimicking the situation in humans, the development of particularly large follicles allows the possibility of collecting large amounts of mural granulosa cells (GC) issuing from the follicular wall as well as large amounts of follicular fluid (30 to 50 ml/follicle) that may be used for OMICS studies in a 1:1 comparison with the developmental status of the enclosed oocyte. The cumulus-oocyte complex (COC)

of the mare is particularly large, thus allowing a 1:1 evaluation of biological parameters of cumulus cells (CCs) predictive of oocyte meiotic and developmental competence. Moreover, follicular COCs in the mare can be recovered from immature follicles, with initial COC morphological features indicating viability (compact cumulus) or atresia (apoptosis, expanded cumulus) of their surrounding follicle, thus supporting study of the effects of follicle immaturity and early or late atresia on oocyte competence (Hinrichs and Williams, 1997; Dell'Aquila et al., 2003). The equine oocyte is approximately 200 microns in diameter, with good visibility of the perivitelline space and the first polar body (PB), so that mature oocytes are easily identified on morphological examination. The horse oocyte possesses a unique distribution of cytoplasmic lipid droplets, which assume polar aggregation in metaphase II (MII) oocytes, and whose biological meaning is under investigation (Ambruosi et al., 2009). Horses make a valuable model for oocyte assessments associated with penetration of the zona pellucida (e.g. PB biopsy) because, in contrast to species such as cattle and sheep, methods for fertilization via intracytoplasmic sperm injection (ICSI) are well established in the horse (Hinrichs et al., 2005; Choi et al., 2006). Sperm injection is necessary to achieve fertilization after penetration of the zona pellucida for investigative purposes, as the defect in the zona would lead to polyspermy if standard in vitro fertilization (IVF) were to be performed. These features make the equine oocyte a particularly useful model for the establishment of OMICS strategies that could be not only applied to better understanding of human assisted reproductive medicine, but also directly applicable to the horse industry.

2.2 Oocyte genomics – The polar body biopsy and genomic analysis for predicting half of the DNA constitution of an embryo: from FISH to CGH/CNV/SNP-based arrays

The genomic DNA constitution of the oocyte determines the sequence of produced transcripts and proteins, and constitutes half of the early embryo phenotype. The most widely used diagnostic tool for oocyte and embryo genomic investigation to date has been Fluorescent In Situ Hybridization (FISH), a cytogenetic technique which identifies specific DNA sequences on chromosomes by means of fluorescent probes that bind to those parts of the chromosome with which they show a high degree of sequence similarity.

Since the first report in humans (Griffin et al., 1992), several studies have been published reporting the evaluation of human day 3 embryos (4-8 cell stage) for up to 8 pairs of chromosomes (chromosomes 13, 15, 16, 17, 18, 21, 22, and X/Y; review by Seli et al., 2010). However, in recent meta-analysis studies, reported from 2008 to 2010, it has become clear that preimplantation genetic screening by using FISH is not justified. This is because it causes damage to the embryo, it requires embryo cryopreservation and transfer in a subsequent cycle, and it does not significantly contribute to the identification and exclusion of aneuploid embryos. Use of FISH has been reported to be associated with lower implantation rates and it shows errors such as false positives due to mosaicism or false negatives due to the limited number of chromosomes analyzed and the limited targeted regions. Therefore, authoritative scientific committees, including the European Society of Human Reproduction and Embryology (ESHRE) and the American Society of Reproductive Medicine (ASRM), decided to conduct a study to determine whether biopsy of the first and second polar bodies (PBs) of the oocyte would enable the timely identification of the chromosomal status of an oocyte. This aim could be reached by analyzing the complete

chromosome complement of the two PBs by Comparative Genomic Hybridization (CGH; reviews by Seli et al., 2010; Geraedts et al., 2010).

Conventional CGH, initially developed by cancer biologists, was applied to human early embryos around 10 years ago (Voullaire et al., 2000 reviewed by Geraedts et al., 2010). These authors performed CGH in association with whole genome amplification by degenerate oligonucleotide-primed polymerase chain reaction. CGH is a competitive hybridization of two fragmented genomes (test and reference genomes) to the chromosomes of a metaphase plate of a normal subject. The tested and subject genomes are labeled with different (red and green) fluorescent dyes so that an increase of red staining will indicate the presence of duplicated regions, an increase of green staining will indicate the presence of deleted regions, whereas the lack of predominance of one of the two colors will indicate normal chromosome structure. This technique allows examination of the whole chromosomal complement, but requires extensive time to get the results. In recent years, CGH-microarray tools have been developed in which the labeled DNAs are affixed to DNA on a microscope slide rather than to metaphase chromosomes. A variety of microarray-CGH platforms are available. As an example, the Cambridge-based company BlueGnome offers an array-based CGH protocol which allows analysis of biopsied PBs within 11 hrs (SurePlex amplification protocol; 24sure analysis, BlueGnome; "http://www.bluegnome.co.uk/"; Geraedts et al., 2010). As regard, Geraedts et al., (2011) and Magli et al., (2011) reported clinical results and technical aspects of a proof-of-principle study performed in associated ART centers in which all mature metaphase II oocytes from patients who consented to the study, fertilized by ICSI, were analyzed. The first and second PBs were biopsied and analysed separately for chromosome copy number by array CGH. If either or both of the PBs were found to be aneuploid, the corresponding zygote was then also processed by array CGH for concordance analysis. It was concluded that the ploidy of a zygote can be predicted with acceptable accuracy by array CGH analysis on both PBs. Interestingly, on the male side, the application of CGH arrays to single human sperm cells has been recently reported (Antonello et al., 2011).

In the aim to move from chromosomal structure to single mutation analysis, SNP (Single Nucleotide Polymorphism) arrays have been developed. A single-nucleotide polymorphism (SNP, pronounced snip) is a DNA sequence variation occurring when a single nucleotide — Adenine (A), Thymine (T), Cytosine (C), or Guanine (G) — differs in a sequence between members of a species or paired chromosomes in an individual. Many common SNPs have only two alleles. Within a population, SNPs can be assigned a minor allele frequency — the lesser of the two allele frequencies for a population. There are variations among human populations, so a SNP allele that is common in one geographical or ethnic group may be rare in another. Unlike the CGH-microarray platforms which involve simultaneous hybridization of differentially labeled DNAs to the same microarray, SNP-microarrays assess test and reference samples, separately, in parallel. From 10.000 to 500.000 SNPs may be evaluated simultaneously. For example, using the Affymetrix platform, analysis of 250.000 SNPs in first PB biopsies (Treff et al., 2010a) and in Day-3 embryos (Treff et al., 2010b) have been reported (review by Seli et al., 2010). The Illumina platform allows the analysis of 370.000 human SNPs.

Because of their utility in recognizing variations associated with disease, recent genetic epidemiology studies have been dominated by genome-wide association studies using

SNPs. However, another form of structural genomic variation, termed copy number variation (CNV), is also widespread throughout the genome. These genomic structural variations range from 1 to 5 Mb and can be highly polymorphic between individuals, and thus can be used for epidemiological study. CNVs in the form of large-scale insertions and deletions, as well as inversions and translocations, may have important roles in meiotic recombination, human genome evolution and gene expression. Many genetic diseases are based on CNVs. However, because they consist of quantitative rather than qualitative changes, show variability in copy numbers and are confounded by the diploidy of the human genome, the detailed genetic structure of CNVs cannot be readily studied by available techniques. Thus, the establishment CNV-microarrays is currently under investigation. New microarray-based technologies will enable more accurate mapping of CNVs, and CNV maps of the human genome are being refined with increasing resolution. The study of CNVs and their effects on human health and disease therefore present a dynamic and exciting challenge for researchers in the field of genetic epidemiology (Wain and Tobin, 2011). The importance of CNVs in human preimplantation genetic screening, or to animal oocyte and embryo testing, has not been reported to date.

Although there is potential economic interest for the application of PB biopsy and subsequent analysis of the chromosomal complement or genome by CGH-, CNV- or SNP arrays in animal husbandry and breeding, to the best of our knowledge no studies have been published to date in large animals. The field is therefore open to future investigations, pending the establishment of the different genomic arrays in these species. A recent report (Le Bourhis et al., 2011) presented for the first time bovine embryo biopsy and genotyping using a 50K SNP Illumina chip. In this study, biopsies of 5 to 10 cells were obtained from *in vitro*-cultured morulae and blastocysts and kept frozen or at room temperature. The genomic DNA of each biopsy was amplified by using a whole-genome amplification kit and was genotyped using a custom CRV 50K Illumina chip. Call rates were calculated from 50.905 SNPs. Percentage of allele drop-out was estimated from the number of heterozygous markers present [% allele drop-out = (calculated heterozygous–observed heterozygous) /calculated heterozygous]. Parentage error was estimated by using the genotypes of the parents of the embryos. A greater quantity of DNA was obtained after amplification of biopsies that were sent frozen to the laboratory than from those at room temperature ($P<0.05$). However, the SNP call rate, % allele drop-out, and parentage error did not differ between groups. These results indicate that genotyping from embryo biopsies following whole genome amplification can be achieved with good efficiency when using high-density marker chips. To the best of our knowledge no studies have been reported to date on genomic analysis in the equine oocyte. A recent paper in the horse by Choi et al., (2010) reports the identification of disease-causing mutations in trophoblastic biopsies from equine in vivo-recovered pre-implantation embryos. These authors demonstrated for the first time the correct identification, by embryo biopsy and whole genome amplification, of sex and genotype at the causative mutation sites for two disease-linked genes (SCN4A and PPIB). The biopsies were performed on Day-6 and Day-7 equine embryos, and after biopsy these embryos were able to produce pregnancies leading to term delivered, normal foals. These two recent studies demonstrate that OMICS technologies have the potential in animal breeding for both marker-assisted selection and for preimplantation diagnosis of genetic diseases.

Another promising genomic investigation area is Epigenetics, the study of changes in gene expression and thus cellular phenotype caused by mechanisms other than changes in the underlying DNA sequence – hence the name *epi-* (Greek: *επι-* over, above, outer) *genetics*. Examples of such changes are DNA methylation and deacetylation of the histones, the proteins around which DNA are wrapped. Both of these changes serve to suppress gene expression without altering the sequence of the silenced genes. These changes may remain through cell divisions for the remainder of the cell's life, and some epigenetic changes in germ cells may potentially last for multiple generations. Epigenetic changes in eukaryotic biology are the basis of the process of cell differentiation. During embryonic morphogenesis, the totipotent cells of the zygote become the various pluripotent cell lines of the embryo, which in turn become fully differentiated cells. This is accomplished by activating some genes while inhibiting others. Current epigenetic research focuses on chromatin modifications occurring during sequential phases of fertilization (sperm chromatin decondensation, pronuclear formation with DNA duplication and syngamy) and early development (chromosome condensation and assembly in the first metaphase plate of the first mitotic division and the subsequent series of mitotic divisions to the blastocyst stage; Burton & Torres Padilla, 2011). These events may be studied by comparing embryos produced in vivo with those obtained using different technologies, such as IVF, ICSI, parthenogenesis or somatic cell nuclear transfer (Cremer & Zakhartchenko, 2011). Soon, the emergence of quantitative high-throughput microarray technology should allow the development of epigenomic arrays for the evaluation of embryo whole-genome epigenetic status, thus opening the new field of epigenomics (Callinan & Feinberg, 2006) to the study of oocyte and embryo competence.

The methylation pattern of DNA in oocytes may be a key factor for the improvement of efficiency of in vitro embryo production, because it is related to oocyte competence. A recent study (Simarro Fagundes et al., 2011) reported on a differentially-methylated region located in exon 10 of the imprinted gene *IGF2* This study evaluated immature vs *in vitro*-matured bovine oocytes from small (1–3 mm in diameter) and large follicles (≥ 8.1 mm in diameter). It was observed that after IVM, oocytes from ≥ 8.1 mm follicles were less methylated (18.51%) than were those from 1- to 3-mm follicles (49.62%). As oocytes from the larger follicles are more developmentally competent, the less methylated pattern appears to be associated with higher oocyte quality. It was concluded that the methylation pattern of specific genes could be used as a molecular marker for epigenetic reprogramming status in oocytes, helping the development of new in vitro embryo production protocols. A broader study on this wave (Smallwood et al., 2011) reported the first integrated epigenomic analysis of mammalian oocytes (GV vs MII oocytes) and preimplantation embryos (blastocyst stage) identifying over a thousand CG islands methylated in matured oocytes. The authors observed that CG islands were preferentially located within active transcription units, supporting a general transcription-dependent mechanism of methylation, and that very few CG islands were protected from post-fertilization reprogramming, the majority showing incomplete demethylation in Day-3 blastocysts. This study revealed the extent and dynamics of CG island methylation in oocytes, which is a prerequisite for defining the full repertoire of imprinted genes and the mechanistic basis of parent-of-origin expression effects in somatic tissues.

Epigenomic studies have not been reported to date in equine oocytes; however, in the promising field of genomic investigations, the equine oocyte would serve as an excellent

model for the comparison of oocyte (metaphase plate) and polar body genomes and epigenomic modifications, due to the ability to investigate the developmental competence of biopsied equine oocytes after fertilization via ICSI.

2.3 Oocyte transcriptomics – The global analysis of oocyte mRNA transcripts

In addition to the genomic constitution of the embryo, it is necessary to know its phenotype: which genes are being utilized at this particular stage of development? Initially, embryonic phenotype is determined by those mRNAs already transcribed and present in the oocyte cytoplasm at the time of fertilization (maternal mRNAs). Evaluation of maternal mRNA content is particularly attractive in the study of developmental biology and for diagnostic and applied purposes in ART (e.g. nuclear reprogramming in cloning, and stem cell research). However, transcriptome analysis of mammalian oocytes and embryos faces three main challenges: 1) the small amount of material available; 2) differing total RNA content in the subsequently-occurring developmental stages, making comparison among stages difficult; 3) existence of oocyte-specific genes often absent from commercially available microarrays (Dalbies-Tran and Mermillod, 2003; Thelie et al., 2009).

Via transcriptomics, it is possible to thoroughly investigate the functional status of a cell line or tissue. Rapidly developing methods consist of RNA extraction, reverse transcription (RT), amplification and labeling, array hybridization, chip scanning, and data interpretation by bioinformatic analysis with subsequent validation by Real Time RT-PCR. Detailed reviews of microarray analysis strategies and interpretation of transcriptomic profiles have been presented by White and Salamonsen, 2005 and Rodriguez-Zas et al., 2008. Some of the public or commercially available software commonly used for trascriptome analysis are:

Public:

- GENE ONTOLOGY: http://www.geneontology.org;
- NCBI Entrez Gene: http://www.ncbi.nlm.nih.gov.sites/entrez?db=gene);
- NCBI Gene Expression Omnibus GEO: http://www.ncbi.nlm.nih.gov/geo;
- KEGG pathway database: http://www.genome.jp/kegg/pathway.html;

Commercially available:

- INGENUITY pathway analysis http://www.ingenuity.com;
- PANTHER Applied Biosystem http://www.pantherdb.org);
- AFFYMETRIX (http://www.affymetrix.com/products/arrays/specific/bovine.affx).

A major aim in oocyte transcriptomics is the analysis of differences among maturation stages, especially between the germinal vesicle (GV) and the metaphase II (MII) stage, as well as differential expression between in vivo- (in vivo-MII) and in vitro- (IVM-MII) matured oocytes. The correct molecular control of meiotic maturation is a fundamental prerequisite for successful development of an early embryo (Tosti & Boni, 2011). Transcriptome microarray technologies have been developed, first in the mouse and more recently in large animals (review by Thelie et al., 2009). At the moment, cattle take center stage in the cast of large animals used as models for human reproductive medicine.

2.3.1 Studies in bovine oocytes

GV vs MII oocyte A pioneering study on oocyte gene expression was conducted at INRA (France) by Dalbies-Tran and Mermillod, in 2003. These authors analyzed gene expression in bovine oocytes before and after IVM, using heterologous hybridization onto a cDNA array. Total RNA was purified from pools of over 200 oocytes either immediately after aspiration from follicles of slaughterhouse cow ovaries, or following IVM. Radiolabeled cDNA probes were generated by RT followed by linear PCR amplification and were hybridized to Atlas human cDNA arrays. To the best of our knowledge, this was the first report of gene expression profiling by this technology in the bovine oocyte. The results demonstrated that cDNA array screening is a suitable method for analyzing the transcription pattern in oocytes, as about 300 identified genes were reproducibly shown to be expressed in the bovine oocyte. The relative abundance of most messenger RNAs appeared stable during IVM; however, it was observed that 70 transcripts underwent a significant differential regulation between meiotic stages (by a factor of at least two). Information obtained in this study constituted the first molecular signature of oocyte cytoplasmic maturation.

GV oocyte vs embryo In a subsequent study at INRA (Thelie et al., 2009) results of an RNA-amplification protocol for bovine oocytes and blastocysts was reported. Using RT-PCR, these authors confirmed that the profiles of both abundant and scarce polyadenylated transcripts were conserved after RNA amplification. Next, amplified probes generated from immature oocytes, in vitro-matured oocytes, and in vitro-produced hatched blastocysts were hybridized onto an in-house cDNA macroarray that included oocyte-specific genes (934 expressed sequence tags of interest including markers of oocyte maturation; Thelie et al., 2009). Following an original approach, two normalization procedures, based on either the median signal or an exogenous standard, were compared and the expected difference in sets of differential genes, depending on the normalization procedure, were calculated. Using a 1.5-fold threshold, no transcript was found to be up-regulated when data were normalized to an exogenous standard, which reflects the absence of transcription during oocyte IVM. In blastocysts, the majority of genes found to be preferentially expressed in oocytes (after normalization) were not activated. This study shed new light on and complemented previous transcriptomic analyses of the bovine oocyte-to-embryo transition using commercial platforms (i.e.: Misirlioglu et al., 2006; Fair et al., 2007; reviewed by Thelie et al., 2009).

In vivo-MII vs IVM-MII oocyte The differences in the MII oocyte transcriptome between oocytes matured in vivo and in vitro were investigated in cattle by Katz-Jaffe et al., (2009). In this study, the Affymetrix Gene Chip Bovine Genome Array, a platform containing over 23.000 bovine transcripts, was used. Transcripts identified as being differentially expressed between the two groups were classified according to gene ontology. Statistical analysis of microarray data identified several processes affected by IVM, including metabolism, energy pathways, cell biogenesis and organization, and cell growth and maintenance. In particular, it was found that 4 genes of the tricarboxylic acid cycle and 14 genes of oxidative phosphorylation were down-regulated in IVM-MII compared with in vivo-MII.

GV vs MII oocyte Mamo et al., (2011) used the Affymetrix GeneChip Bovine Genome Array to perform global mRNA expression analysis of immature (GV) and in-vitro matured (IVM) bovine oocytes. They then used a variety of approaches, including the analysis of transcript abundance in oocytes matured in the presence of alpha-amanitin (a transcription inhibitor),

to determine whether the transcriptional changes observed during IVM were real or were artifacts of the techniques used during analysis. It was found that 8489 transcripts were detected across the two oocyte groups, of which ~25.0% (2117 transcripts) were differentially expressed ($p<0.001$); corresponding to 589 over-expressed and 1528 under-expressed transcripts in the IVM oocytes compared to their immature counterparts. Subsets of the differentially expressed genes were validated by quantitative RT-PCR and the gene expression data was classified according to gene ontology and pathway enrichment. Numerous cell-cycle linked (CDC2, CDK5, CDK8, HSPA2, MAPK14, TXNL4B), molecular transport (STX5, STX17, SEC22A, SEC22B), and differentiation (NACA) related genes were found to be among the over-expressed transcripts in GV oocytes compared to their mature counterparts, while other genes (ANXA1, PLAU, STC1and LUM) were among the over-expressed genes after maturation. This data set provided a unique reference resource for studies of the molecular mechanisms controlling oocyte meiotic maturation in cattle, and by extension to other species, and through use of the alpha-amanitin, addressed the existing conflicting issue of transcription during meiotic maturation.

Adult vs prepubertal oocyte (Romar et al., 2011) This study, rather than applying global OMICS strategies, analyzed the differential expression profile of adult vs prepubertal bovine oocytes by using a specialized panel for genes involved in the maturation process, such as genes known to specifically affect early development after fertilization (maternal-effect genes, shown via mouse knock-outs), biomarkers of oocyte competence or redox metabolism, or genes involved in the regulation of meiotic progression. It was found that some genes (particularly redox genes) are significantly underexpressed in oocytes from prepubertal subjects. This kind of comparison would benefit greatly by using OMICS technologies and underscores the value of animal models, as it would be difficult to perform in humans due to low availability of oocytes from young girls and women.

2.3.2 Studies in human oocytes

Transcriptomic studies have been reported in human oocytes. Kocabas et al., 2006 reported the use of a comprehensive human microarray platform (Affymetrix Human Genome U133 Plus 2.0 GeneChips) to identify the gene transcripts present in **early MII oocytes**, tested within minutes after isolation from the ovary.

In the study by Wells and Patrizio (2008), unfertilized **GV**, **in vivo-MII** and **IVM-MII** oocytes were analyzed. The study used the Applied Biosystem Human Genome Survey Microarray with 32.878 60mer oligonucleotide probes for the interrogation of 29.098 genes, including 8000 genes not previously included in any commercial array. By bioinformatic analysis, a Venn diagram can be obtained in which each circle represents the transcriptome of a specific cell type, and overlapping areas indicate commonly-expressed genes. The three oocyte categories expressed 12.219, 9.735 and 8.510 genes, respectively. There were extensive overlaps among the three groups, but also some significant differences. In particular, in vivo-MII and IVM-MII oocytes shared similar patterns of gene expression. However, some immature patterns of expression, reminiscent of GVs, persisted in IVM-MIIs. In humans, in-vitro maturation is an attractive strategy for IVF treatment; however, currently IVM oocytes perform poorly after IVF. Data from the this study indicates that although IVM-MII oocytes closely resemble in vivo-MII oocytes in cellular pathways related to nuclear maturity, several pathways associated with cytoplasmic functions continue to be expressed in an

immature manner. Additionally, it was shown that IVM-MII oocytes differ in the expression of genes related to cellular storage and homeostasis. Such differentially expressed genes and their pathways provide clues for the optimization of IVM techniques, and, importantly, a method to assess the effects of those techniques on oocyte competence without having to evaluate development after fertilization, which could raise ethical issues in humans.

In vivo-MII vs IVM-MII In the study by Jones et al., (2008), more than 2000 genes were identified as expressed at more than 2-fold higher levels in oocytes recovered from gonadotropin-stimulated cycles and matured in vitro than those matured in vivo, and 162 of these were expressed at 10-fold or greater levels (this study used Codelink Whole Human Genome Bioarrays, GE Healthcare Biosciences). It was concluded that the overabundance of transcripts in immature oocytes recovered from gonadotropin stimulated cycles, then matured in vitro could be due to dysregulation of either gene transcription or post-transcriptional modifications, resulting in incorrect temporal utilization of genes, culminating in developmental oocyte incompetence.

GV vs IVM-MII vs embryo Zhang et al., 2009a followed the transcriptome changes occurring in human preimplantation development by applying microarray analysis (Affymetrix two-cycle GeneChip Eukaryotic small sample target labeling assay version II; HG-U133 Plus 2.0 array) to human oocytes and embryos at six developmental stages. They observed a dramatic reprogramming of transcription and translation during preimplantation development in a stage-specific manner, with two main transitions (MII to Day 2 and Day 3 to Day 5). Over 47.000 transcripts expressed in oocytes and early embryos were reported, thus providing a fundamental resource for understanding the genetic control of human early development. There was a significant underrepresentation of transcripts responsible for cell signaling and communication (genes associated with the G protein coupled receptor - GPCR - protein signaling pathway, cell communication, immune response, response to external stimuli, cell adhesion, sensory perception and cell-surface receptor-linked signal transduction pathways) in both oocytes and embryos, when compared to adult tissue; the authors concluded that human preimplantation development is almost self-directed -- i.e., oocytes and embryos apparently do not need to communicate with the "external world" to the same degree as adult tissues do. This paper also performed evolutionary comparisons between humans and mice, dogs and chimpanzees. Genes that were highly expressed in human oocytes and embryos varied less from those of other species than did genes of adult tissues: the conclusion was that these "pre-implantation genes" are highly conserved.

Microarray analysis of human oocytes has been subsequently applied to a variety of reproductive issues. Wood et al., (2007) found differences in gene expression between **normal** and **PCOS** (polycystic ovarian syndrome) oocytes for 8123 transcripts, 374 of which were genes related to meiotic spindle dynamics. Grondahl et al., (2010), evaulated 15 independent replicates of single **in vivo-MII** oocytes using the Affymetrix HG-U133 Plus 2.0 gene chip array, which tests around 48.000 well identified genes by using around 56.000 probe sets, and the Affymetrix gene array 2500 scanner. These authors identified 7.470 genes (10.428 transcripts) as present in human in vivo-MII oocytes. Of these, 342 genes showed a significantly different expression level between **young** and **aged** women; notably, genes annotated to be involved in cell cycle regulation, chromosome alignment (e.g. MAD2L1 binding protein), sister chromatid separation (e.g. separase), oxidative stress and

ubiquitination. The top signaling network affected by age was 'cell cycle and organism development' (e.g. SMAD2 and activin B1 receptor). Thus, this study provided information on processes that may be associated with lowered oocyte developmental competence due to ageing.

2.3.3 The common gene expression signatures of oocytes and embryonic stem cells

Another interesting research area for transcriptomic investigations is the comparison of the transcriptomic profile between MII oocytes and stem cells. Data have been published on the comparison between human **MII oocytes** and human **embryonic stem cells (ESC)**. Both of these cells types are able to reprogram differentiated nuclei towards pluripotency, either by somatic cell nuclear transfer or by cell fusion, respectively. Comparison of the transcriptome of these two cell types may highlight genes that are involved in induction of pluripotency. Based on a microarray compendium of 205 samples, Assou et al., 2009 compared the gene expression profile of MII oocytes and human ESC to that of somatic tissues. A common oocyte/hESC gene expression profile was identified, which included a strong cell cycle signature, genes associated with pluripotency such as *LIN28* and *TDGF1*, a large chromatin remodelling network (*TOP2A, DNMT3B, JARID2, SMARCA5, CBX1, CBX5*), 18 different zinc finger transcription factors, including *ZNF84*, and several still-poorly annotated genes such as *KLHL7*, *MRS2*, and Selenophosphate synthetase 1 (*SEPHS1*). Interestingly, a large set of genes in both cell types was found to code for proteins involved in the ubiquitination-proteasome pathway. Upon ESC differentiation into embryoid bodies, the transcription of genes in this pathway declined. In vitro, a selective sensitivity of human ESC to inhibition of proteasome activity was observed. These results shed light on the gene networks that are concurrently overexpressed by the two cell types with somatic cell reprogramming properties.

2.3.4 Prediction of oocyte competence based on analysis of accessory cells (polar bodies, cumulus cells or granulosa cells)

A major problem of reproductive biotechnologies is predicting which oocytes are destined to develop into viable embryos. Analysis of accessory cells, such as PBs, CCs and GCs, allows oocyte quality assessment without interfering with use of the oocyte in ART.

Polar Body Klatsky et al., (2010) reported detection and quantification of mRNA from single human polar bodies, a minimally invasive test of the oocyte gene-expression profile. Gene expression of 12 candidate genes was investigated in PB biopsies and the oocytes from which they originated, and polar-body mRNA was detected for 11 out of 12 genes. This method would allow detection and comparison of individual differences in oocyte gene expression without harming the oocyte.

Granulosa cells The comparative evaluation of the effects of FSH vs human menopausal gonadotrophin on GCs has been reported (Grondahl et al., 2009). These authors found that the drugs used for controlled ovarian hyperstimulation have a significant impact on the gene expression profile of human granulosa cells. Interesting differences were observed for genes involved in the regulation of preovulatory events. For GC in the mare, see the work by Fahiminiya et al., 2010 in section 2.3.5

Cumulus cells The bi-directional communication between the oocyte and its companion CCs is crucial for the development and function of both cell types. Regassa et al. (2001) investigated the transcripts that are exclusively expressed either in oocytes or in CCs, and the molecular mechanisms affected when communication between the two cell types is removed. The transcriptomic profile of different oocyte and CC samples was analyzed by using Affymetrix GeneChip Bovine Genome array containing 23000 transcripts. Out of 13162 genes detected in GV oocytes and their companion CCs, 1516 and 2727 were exclusively expressed in oocytes and in CCs, respectively, while 8919 were expressed in both. Similarly, of 13602 genes detected in MII oocytes and CCs, 1423 and 3100 were exclusively expressed in oocytes and in CCs, respectively, while 9079 were expressed in both. A total of 265 transcripts were differentially expressed between oocytes cultured with (OO+CCs) and without (OO-CCs) CCs, of which 217 and 48 were over-expressed in the former and the latter groups, respectively. Similarly, 566 transcripts were differentially expressed when CCs were cultured with (CCs+OO) or without (CCs-OO) their enclosed oocytes. Of these, 320 and 246 were over-expressed in CCs+OO and CCs-OO, respectively. While oocyte-specific transcripts include those involved in transcription (IRF6, POU5F1, MYF5, MED18) and translation (EIF2AK1, EIF4ENIF1), CC-specific transcripts include those involved in carbohydrate metabolism (HYAL1, PFKL, PYGL, MPI), protein metabolic processes (IHH, APOA1, PLOD1) and steroid biosynthetic process (APOA1, CYP11A1, HSD3B1, HSD3B7). Similarly, while transcripts over expressed in OO+CCs were involved in carbohydrate metabolism (ACO1, 2), molecular transport (GAPDH, GFPT1) and nucleic acid metabolism (CBS, NOS2), those over expressed in CCs+OO were involved in cellular growth and proliferation (FOS, GADD45A), cell cycle (HAS2, VEGFA), cellular development (AMD1, AURKA, DPP4) and gene expression (FOSB, TGFB2). This study generated large-scale gene expression data that provide insights into gene function and interactions within and across different pathways that are involved in the maturation of bovine oocytes. Moreover, the presence or absence of oocyte and CC factors during bovine oocyte maturation has a profound effect on transcript abundance in the different cell types, showing the important molecular cross-talk between oocytes and their CCs. This kind of study has not yet been performed in humans.

A more recent study reported the transcriptomic analysis, by using the Affymetrix Bovine Expression Array, of granulosa cells and oocytes from newborn sheep ovaries (primordial, primary, secondary and small antral follicles) isolated by Laser Capture Microdissection (Bonnet et al., 2011). This study will significantly support clinical programs for rescue of fertility (oocyte production potential) in young women affected by ovarian pathologies or undergoing cancer therapy.

2.3.5 Preliminary data and prospective use of equine oocytes

The equine oocyte would make a valid animal model for transcriptomic studies of predictive markers of oocyte quality via analysis of PBs, CCs and GCs. Its peculiarly large PB size and the unique opportunity, due to large follicle size, to perform 1:1 oocyte:somatic cell ratios could allow reliable identification of predictive parameters of oocyte competence by analyzing the PB, CC or GC transcriptome.

Recently, molecular studies preliminary to OMICS applications have been performed in equine embryos. Paris et al., (2011) identified and validated a set of **reference genes** suitable

for studying gene expression during equine embryo development. The expression of four carefully-selected reference genes and one developmentally-regulated gene was examined by quantitative PCR in equine in vivo-produced embryos, from the morula to the expanded blastocyst stage. SRP14, RPL4 and PGK1 were identified by geNorm analysis as stably-expressed reference genes suitable for data normalisation. RPL13A expression was less stable and changed significantly during the period of development examined, rendering it unsuitable as a reference gene. As anticipated, CDX2 expression increased significantly during embryo development, supporting its possible role in trophoectoderm specification in the horse. In summary, it was demonstrated that evidence-based selection of potential reference genes aids in validation of stable gene expression in an experimental system, which is particularly useful when dealing with tissues that yield small amounts of mRNA.

Smits et al., (2011) evaluated the difference between **in vivo-** and **in vitro-produced** (IVP) **equine blastocysts** at the genetic level. Suppression subtractive hybridization (SSH) was used to construct a cDNA library enriched for transcripts preferentially expressed in in vivo-derived equine blastocysts compared with IVP blastocysts. Of the 62 different genes identified in this way, six genes involved in embryonic development (BEX2, FABP3, HSP90AA1, MOBKL3, MCM7 and ODC) were selected to confirm this differential expression by RT-quantitative PCR. Five genes were confirmed to be significantly upregulated in in vivo-derived blastocysts (FABP3, HSP90AA1, ODC, MOBKL3 and BEX2), confirming the results of the SSH, however, there was no significant difference in MCM7 expression. Because of their possible importance in embryonic development, the expression of these genes can be used as a marker to evaluate in vitro embryo production systems in the horse, and can be used to compare their roles in embryo development of other species.

Previous studies of functional transcriptomics of individual or associated gene sequences in the **equine oocyte** have been reported by our laboratory (Dell'Aquila et al., 2004 for connexin 43, cyclooxygenase-2 and FSH receptor; Caillaud et al., 2009 for interleukin 1β and its receptors; Dell'Aquila et al., 2008 for the mu opioid receptor; De Santis et al., 2009 for the extracellular calcium-sensing receptor; Lange Consiglio/Cremonesi et al., 2009 for leptin and its ObR receptor) and other groups (Lindbloom et al., 2008 for EGF-like growth factors; Lupole et al., 2010 for ZP genes). To the best of our knowledge, few studies have been performed to date with OMICS technologies in equine reproductive cells or tissues. Fahiminiya et al., (2010) investigated the transcriptome of **granulosa** and **theca cells** from equine follicles at different developmental stages. An equine gene-expression microarray (Agilent technologies Inc., CA, USA) with 44.000 probes was used. Cells were examined from early dominant vs late dominant follicles, and from preovulatory follicles 34 h after injection of crude equine gonadotrophin. It was found that 8349 transcripts were differentially expressed in GC and 2338 in theca cells between preovulatory and late dominant follicles, and that 1602 transcripts were differentially expressed in GC and 8 in theca cells in late dominant vs. early dominant follicles. Thus, it appears that the GC have a highly dynamic nature during the development of dominant follicles. In additional work, Das et al. (2010) analyzed **sperm** and **testis** transcriptomes using the Texas A&M equine whole genome 21351-element oligoarray. Bruemmer et al. (2010) analyzed the **endometrium** transcriptome using the Horse gene expression Agilent microrray for 43000 transcripts. Slough et al., (2011) studied the gene expression (StAR, 3β-HSD, cox, and caspase-3) profile of equine corpus luteum tissue recovered by in vivo biopsy.

2.4 Oocyte proteome: The direct representation of the oocyte phenotype

Oocyte mRNA is not a direct representation of the factors that drive oocyte phenotype. The identified mRNA represent potential proteins, but the degree to which the mRNA are being translated is unknown. Thus, an OMICs goal is the identification and measurement of all proteins expressed in the oocyte or embryo. There are two main protein-containing oocyte compartments, the cytoplasm and the zona pellucida. The protein makeup of the oocyte is important for more than simply evaluating oocyte viability; as noted above, the mammalian oocyte cytoplasm possesses factors which can reprogram terminally-differentiated germ cells (sperm) or somatic cells within a few cell cycles. Moreover, it has been suggested that use of oocyte-derived transcripts may enhance the generation of induced pluripotent stem cells. The zona pellucida is composed of glycoproteins involved in the sperm-oocyte interactions which modulate sperm penetration and the fertilization process. Thus, improving our knowledge of oocyte global protein composition is of great interest.

The main phases of proteomic analysis consist of protein extraction, digestion, separation of proteins by gel- or non gel-based methods, mass spectrometry evaluation of digested and separated or electrosprayed peptides, and bioinformatic data analysis (for detailed proteomic methods, see Seli et al., 2010; Wang et al., 2010 and Arnold & Frohlich, 2011). A major problem in oocyte proteome analysis is the requirement of large numbers (thousands) of oocytes. This problem has limited the possibility of performing studies in the human; studies performed to date have been conducted in the mouse and in large animals.

2.4.1 Proteomic studies in the mouse oocyte

Studies in the mouse have incorporated qualitative proteomic approaches, with the intention of generating a protein database to be used for the molecular characterization of the oocyte and developing embryo.

In a pioneering study by Meng et al. (2007), proteomic profiling of mouse mature **COCs** was performed, using two-dimensional electrophoresis and mass spectrometry. A total of 259 protein spots were identified, corresponding to 156 individual proteins. Functional classification of the identified proteins, performed manually according to the biological function of their coding genes, indicated that 12% were involved in cell signaling/communication, 7% in cell division, 31% in gene/protein expression, 24% in cell metabolism, 10% in cell structure and motility, 12% in cell/organism defense, and 4% were unknown.

In a subsequent study by Ma et al., (2008), two-dimensional electrophoresis of mouse **metaphase-II (MII) ooplasmic** proteins (the ZP was removed by digestion before protein extraction) was performed to describe the proteome and phosphoproteome of oocytes derived from ICR mice. A total of 869 selected protein spots, corresponding to 380 unique proteins, were identified successfully by mass spectrometry. Of these, 90 protein spots, representing 53 unique proteins, were stained by Pro-Q Diamond dye, indicating that, within the MII oocyte cytoplasm, they are in phosphorylated forms. All identified proteins were bioinformatically annotated and compared to the embryonic stem-cell proteome. A proteome reference database for the mouse oocyte was established from the protein data generated in this study (http://reprod.njmu.edu.cn/2d).

A subsequent study (Zhang et al., 2009b) applied one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis and reverse-phase liquid chromatography tandem mass spectrometry to analyze the **mature oocyte proteome** of the mouse in depth. Using this high-performance proteomic approach, the authors successfully identified 625 different proteins from 2700 mature mouse oocytes denuded of their zonae pellucidae. They identified 76 maternal proteins having high levels of mRNA expression both in oocytes and fertilized eggs. Many well-known maternal-effect proteins were included in this subset, including MATER and NPM2. In addition, the observed mouse oocyte proteome was compared with a recently published mouse **embryonic stem cell (ESC) proteome** (Van Hoof et al., 2006, see ref. in Zhang et al., 2009)**,** and 371 overlapping proteins were identified.

In a more recent study by Wang et al. (2010), 7,000 mouse oocytes at different developmental stages, including the **GV stage**, the **MII stage**, and **fertilized oocytes (zygotes)**, were evaluated. The authors successfully identified 2,781 proteins present in GV-stage oocytes, 2,973 proteins in MII oocytes, and 2,082 proteins in zygotes, through semiquantitative mass spectrometry. The results of the bioinformatics analysis indicated that different protein compositions were correlated with oocyte characteristics at different developmental stages. For example, specific transcription factors and chromatin remodeling factors were more abundant in MII oocytes, which may be crucial for the epigenetic reprogramming of sperm or somatic nuclei. These results provided important knowledge for better understanding of the molecular mechanisms associated with early development, and may improve the generation of induced pluripotent stem cells.

A more recent study (Pfeiffer et al., 2011) reported the proteome of MII mouse oocytes to a depth of 3699 proteins, which extends the number of proteins identified to date in mouse oocytes to a comparable size to that of the proteome of undifferentiated mouse ES cells. Twenty-eight oocyte proteins, also detected in ES cells, matched the criteria of the multilevel approach reported in this study to screen for reprogramming factors, namely nuclear localization, chromatin modification, and catalytic activity, thus advancing the definition of "reprogrammome", the set of molecules (proteins, RNAs, lipids, and small molecules) that enable nuclear reprogramming.

2.4.2 Proteomic studies in farm animal oocytes

Studies in farm animals, such as cattle and pigs, have been performed for both qualitative database generation and for quantification of proteome changes during oocyte IVM (bovine: Coenen et al., 2004; Bhojwani et al., 2006; Massicotte et al., 2006; Memili et al., 2007; Berendt et al., 2009; pig: Ellederova et al., 2004; Susor et al., 2007, reviewed by Ma et al., 2008; Zhang et al., 2009; Arnold & Frohlich, 2011).

Berendt et al. (2009) performed two-dimensional gel electrophoresis saturation labeling to detect quantitative differences in the proteomes of immature versus IVM-MII **bovine oocytes**. From 250 ng of sample analyzed per gel, quantitative analysis revealed an average of 2244 spots in pH 4–7 images and 1291 spots in pH 6–9 images. Focusing on the pH 4-7 images, 38 spots with different intensities between oocyte stages were detected. Spots on a gel from 2200 immature oocytes were identified by nano-LC-MS/MS analysis. The ten spots which could be unambiguously identified include the translationally

controlled tumor protein, enzymes of the Krebs and pentose phosphate cycles, clusterin, 14-3-3 ε protein and redox enzymes. In addition, the cellular distribution of two differentially-expressed proteins (14-3-3 ε protein, a mediator of Cdc25 phosphatase inhibition, and TCTP, translationally controlled tumor protein) was determined by confocal laser-scanning microscopy. The quantitative and cellular distribution differences of proteins identified in this study may help to identify attractive candidate proteins for oocyte quality evaluation.

To the best of our knowledge, no proteomics studies have been performed to date in the **equine oocyte**. In our group, the functional role of individual proteins involved in the regulation of meiotic maturation has been investigated by means of western blot or immunostaining and confocal microscopy (Dell'Aquila et al., 2008, De Santis et al., 2009; Lange Consiglio et al., 2009). Equine oocytes could be excellent models for oocyte proteomic studies due to the high abundance of maternal proteins accumulated in their large cytoplasm (160 to 180 microns in diameter) during oogenesis. The relatively large cytoplasmic volume is an important feature as it reduces the number of oocytes needed for effective protein extraction, thus increasing the specificity of the proteome analysis. A recent study performed in Xenopus laevis oocytes, chosen due to their abundant ooplasm, identified a number of proteins involved in the regulation of M-phase entry (Kubiak et al., 2011). The equilibrium among activites of these proteins is responsible for the quality of oocytes and the extent of embryo development, via their participation in decision whether to resume meiosis. Identification of cell-cycle control protein activities in mammalian oocytes may have a great impact on the study not only of oocyte quality but also of cancer growth regulation, and thus establishment of targeted therapies.

On the male side, the global proteome of **sperm** and **seminal plasma of fertile stallions** has been investigated (Novak et al., 2010) to determine whether associations exist between the observed proteome and in vivo fertility. Semen was collected throughout the breeding season from 7 stallions at stud in a commercial breeding station. The stallions were bred to a total of 164 mares to determine conception rates. On three occasions during the breeding season, raw semen was obtained from a regular collection and subjected to proteomic analysis using two-dimensional electrophoresis. The semen sample was also assessed for routine semen-quality end points. The first cycle conception rate was negatively related to ejaculate volume ($r = -0.43$, $P = 0.05$) and total IGF1 content (ng) per ejaculate ($r = -0.58$, $P=0.006$), whereas the overall pregnancy rate was positively related to sperm concentration ($r = 0.56$, $P = 0.01$). The abundance of three proteins known to be involved in carbohydrate metabolism in sperm was positively related to fertility. Abundance of cysteine-rich secretory protein 3 (CRISP3) was positively related to first cycle conception rate ($r = 0.495$, $P = 0.027$) and may provide a good marker of fertility. The abundance of four seminal plasma proteins was negatively related to fertility; these were identified as kallikrein-1E2 (KLK2), clusterin, and seminal plasma proteins 1 (SP1) and 2 (SP2). Based on stepwise regression analysis, low levels of clusterin and SP1 in seminal plasma together with abundance of sperm citrate synthase were predictive of fertility ($r=0.77$, $P< 0.0001$). This study identified proteins within sperm and seminal plasma that could serve as biomarkers of semen quality and fertility in stallions, and may present valid models for sperm fertility biomarkers in humans.

2.5 Oocyte lipid fingerprint – Investigating the biological role of structural and reserve lipids

Recently, a new OMIC strategy, namely lipidomics, which utilizes mass spectrometry (MS), chromatography and computer-assisted data analysis, has been proposed. In this approach, lipid molecules are extracted from cells and analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS (Huang et al., 2005). Like other OMICS, lipidomics is a subject which is both technology-driven and technology-driving, allowing changes in lipid metabolism, including the appearance of new species and the disappearance of others, and compartimentalization of specific lipid species, to be investigated. The underlying fundamentals of different lipidomic experimental approaches and the application of these approaches to the identification of inborn errors of metabolism were reported by Griffiths et al. (2011). Maturing mammalian oocytes, particularly those of farm animals, contain large numbers of cytoplasmic lipid droplets (LDs) whose functional role is still under investigation (Ambruosi et al., 2009). Lipid droplets are discrete organelles present in most cell types and in organisms including bacteria, yeast, plants, insects and animals. Long considered as passive storage deposits, recent proteomic and lipidomic analyses show that LDs are dynamic organelles involved in multiple cellular functions. They serve not only as main reservoirs of neutral lipids such as triglycerides and cholesterol but also contain structural proteins, proteins involved in lipid synthesis and transmembrane proteins (review by Kalantari et al., 2010). A recent study (Ferreira et al., 2010) reported the direct lipid analysis by MALDI-MS of single and intact human, bovine, sheep and fish oocytes. Characteristic lipid profiles, mainly represented by phosphatidylcholines, sphingomyelins and triacylglycerols, were obtained. This study demonstrated that MALDI-MS is capable of providing a reproducible lipid fingerprint from a single oocyte and can be used to investigate developmental modifications or the effects of culture conditions. To our knowledge, no lipidomic studies have been reported to date in the equine oocyte. The equine oocyte, being characterized by polar aggregation of cytoplasmic LDs during maturation, could help to significantly clarify the role of LDs in the maturation and fertilization processes, and in early embryonic development.

2.6 Oocyte glycomic analysis – Post-translational protein-carbohydrate modifications

Glycomics deals with the structure and function of glycans or carbohydrates. Lectin-based diagnostics are the main tool aimed at the detection of diseases associated with alterations of the glycosylation profiles of cells. Lectins are proteins that specifically bind to carbohydrates, of either mono- or oligosaccaridic structure. Certain lectins even recognize cell determinants which are not detected by available antibodies. The increasing use of lectins in biomedical diagnostics is leading rapidly to the development of **lectin/glycan microarrays** which could provide efficient, rapid screening tools to detect normal or altered glycosylation patterns in biological samples. Information on glycomics, concerning methods for use of recombinant and artificial lectins and a recently-launched detection platform using lectin microarrays, as well as their application, were reported by Mislovicova et al., 2009 and Gemeiner et al., 2009. A Glycomics DataBase – a data integration platform for glycans and their structures has been recently created (http://www.glycomics.bcf.ku.edu). To our knowledge no studies have been performed to date using large lectin arrays to evaluate oocyte quality. A recent paper by our research unit (Desantis et al., 2009) reported

the use of a 13-lectin panel to evaluate differences of the glycoconjugate pattern between equine oocytes surrounded by compact (viable) or expanded (early atretic) cumulus oophorus. It was found that: 1) equine COCs have a species-specific carbohydrate composition; 2) biosynthesis of glycosylated ZP proteins occurs in both CCs and oocytes; 3) viable (compact) and atretic (expanded) COCs express different lectin-binding patterns in their CCs, ZP and ooplasm. This paper also reviewed numerous studies published on the glycoconjugate pattern of cumulus cells, ZP and ooplasm in several species, including humans. These data confirm that the mare is a good model for evaluation the glycome of oocytes of different quality, developmental stage or functional status, and that the application of lectin arrays could be of great value in evaluating oocyte pathology or the effects of culture conditions.

2.7 Oocyte metabolic profiling – The instantaneous snapshot of oocyte physiology

Following in the wake of OMICS revolutions, new fields of research are emerging. Among them is metabolomics, a field that holds great promise for the study of oocyte and embryo physiology. The metabolome, that is, the compounds produced by the oocyte, provides the natural complement to the genome and proteome. The physicochemical diversity of the metabolome leads to a subdivision of metabolites into compounds soluble in aqueous solutions or those soluble in organic solvents. A complete molecular and quantitative investigation of the latter when isolated from tissue, fluid or cells is a subset of lipidomics (see Section 2.5). A high-priority aim in evaluating oocyte quality is to establish a non-invasive quantification method. Analysis of oocyte metabolism, by evaluating the follicular fluid (FF) or culture media metabolome could be a useful predictor of pregnancy outcome (Sing & Sinclair, 2007; Nel-Themaat & Nagy, 2011). An important aspect limiting this kind of study in humans is the availability of FF aspirated from individual follicles or availability of culture media of individual oocytes. To correlate FF substances with oocyte quality, it is imperative that each follicle is aspirated individually. The procedure of single follicle aspiration is problematic, both for the patient and for the physician, because it requires multiple vaginal punctures (Revelli et al., 2009). Moreover, needle flushing with culture medium after every puncture must be performed, with a standard volume of flushing medium, in order to avoid cross-contamination and to control the dilution of FF substances. In this context, the availability of animal models from which large numbers of individual specimens may be obtained is of great help. A recent review by Revelli et al. (2009) provides an overview on the current knowledge of biochemical predictors of oocyte quality in FF, starting from studies on single biochemical markers and concluding with the most recent studies on metabolomics. Another study (Nagy et al., 2009) evaluated whether near-infrared spectroscopy-generated metabolomic data, obtained from individual oocyte culture media samples, would correlate with nuclear maturity status and subsequent embryo development. Drops (15-20 μl) of in vitro culture media from 3 h culture of individual oocytes recovered from patients undergoing controlled ovarian hyperstimulation were used to obtain a "viability index" of near-infrared spectroscopy metabolomic profiles. Oocytes at different meiotic stages showed significantly different indices, with a higher viability index related to nuclear maturity (MII stage), embryo grade at Day 3 and Day 5 (grade A) and pregnancy rate (human chorionic gonadotropin-positive).

3. Conclusions

OMICS are promising strategies for oocyte quality evaluation in the field of ART, and will lead to a greater understanding of the mechanisms involved in normal oocyte maturation, fertilization and embryo development. It is increasingly clear that large animal models, particularly those species such as horses, whose reproductive management is aimed not only to productivity but also to overcoming infertility, can provide a clinical and biological model for human reproductive phenomena. In addition to their value related to the understanding of human and animal reproduction, "oocyte OMICS" will undoubtedly reveal unexpected and invaluable information that will significantly contribute to the study of stem cell and cancer biology.

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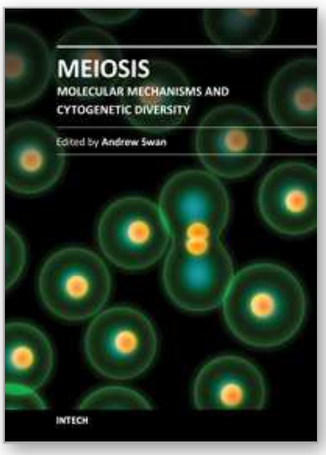

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Meiosis, the process of forming gametes in preparation for sexual reproduction, has long been a focus of intense study. Meiosis has been studied at the cytological, genetic, molecular and cellular levels. Studies in model systems have revealed common underlying mechanisms while in parallel, studies in diverse organisms have revealed the incredible variation in meiotic mechanisms. This book brings together many of the diverse strands of investigation into this fascinating and challenging field of biology.

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