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OMICS-TECHNOLOGIES TO ANALYZE INDIVIDUAL BOVINE OOCYTES FOR IDENTIFICATION OF DEVELOPMENTAL OOCYTE COMPETENCE BIOMARKERS

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Abstract

Oocyte quality is a capacity to be fertilized and to develop into viable embryo and this is crucial for reproductive biotechnologies in farm animals. Technical progress and possible miniaturization of «omics» technologies made possible application of transcriptomic, proteomic and lipidomic methodologies to single oocyte, and thus to search molecular factors representing possible markers of oocyte quality. Oocyte quality is determined by its follicular environment and affects transcript, protein and lipid composition of an oocyte, which has to progress through maturation — a final step before fertilization, crucial for the acquisition of oocyte developmental competence. Here we describe the examples of «omics» analysis performed on single bovine oocytes and their neighboring cumulus cells through the comparison of the oocytes with different competence to mature and to develop blastocyst *in vitro*. In particular, we focus on original technologies of proteomics and lipidomics based on mass spectrometry phenotyping of intact cells and identification of molecular biomarkers.

Keywords: «omics» technologies, single oocyte, bovine

The assessment of gamete quality is important for reproductive biotechnologies, since high quality is essential in conceiving embryos with full development ability. In general, 20-50 % of high-producing lactating dairy cows have already experienced pregnancy loss during the first week of gestation [1]. Although many different factors — like genetic background (race, quantitative trait loci etc), physiological stage (age, parity, lactation...) or environmental impacts (nutrition, temperature stress etc) — can affect the reproductive efficiency in dairy cows, 10-20 % of artificial insemination failure and pregnancy losses could be explained by fertilization failure and early embryo mortality. In modern dairy cow, like Holstein, fertilization rate is typically above 80%, thus early embryonic losses may be linked to compromised oocyte quality due to a poor follicular microenvironment, suboptimal reproductive tract environment for the embryo, and/or inadequate maternal — embryonic communication [2]. Indeed, oocyte maternal factors are at the first line to assure embryo development, before the activation of its proper genome, at the stage of 8-16 cells in cattle.

The understanding of the determinants of oocyte competence to be fertilized and to assure embryo viability during the first cleavages in progress through large-scale analysis of oocyte molecular factors. In the recent decade numerous “omics” technologies, which encompass genomics, transcriptomics, proteomics, metabolomics (including lipidomics) and epigenomics disciplines,

allowed the significant advances in the understanding of oocyte biology [3].

Oocyte acquires its developmental potential inside of the follicle, and the most important stage before an oocyte becomes ready for fertilization, is oocyte maturation. It is now possible to initiate early embryonic development by co-incubating oocytes with treated spermatozoa following a protocol known as *in vitro* fertilization (IVF), which is routinely used in reproductive biotechnologies in cattle. The original process includes the administration of high levels of gonadotropins to stimulate oocyte maturation and ovulation *in vivo*; whereas *in vitro* oocyte can resume meiosis without gonadotropins. Oocyte maturation is a transition from immature germinal vesicle transcriptionally silenced oocyte to metaphase-II stage which is accompanied by polar body extrusion, ooplasm reorganization, and molecular modifications including changes in protein abundance and post-translational modifications [4]. Oocyte maturation plays an important role in the acquisition of oocyte developmental competence, referred as oocyte quality, which at the same time is crucial for embryo quality. Maturation can be accomplished *in vitro* starting from the oocytes recovered from small antral follicles at the stage prior to selection and dominance, by using specific culture media, following a protocol known as *in vitro* maturation (IVM). IVM is promising technique in animal reproduction biotechnology, especially in genetic merit dairy cows, where the success rate after artificial insemination may be low. In cattle, immature oocytes can be obtained from the ovaries either by ovum pick up or after slaughter, then subjected to IVM, fertilized and developed to transferable embryos. Comparing immature and mature oocytes demonstrating contrasted developmental capacity, by using different “omics” technologies helps to reveal molecular factors involved in oocyte quality.

Unlike spermatozoa, the main problem with implementing of “omics” approaches like transcriptomics, proteomics and metabolomics to the female gametes is low biological material content in single oocyte. In bovine full-grown oocyte, total quantities of RNA, protein or lipids are estimated to approximately 2-3 ng, 80-130 ng and 200-250 ng per oocyte, respectively. Nevertheless, significant progress in the adapting of “omics” tools to single oocyte was observed during the last years.

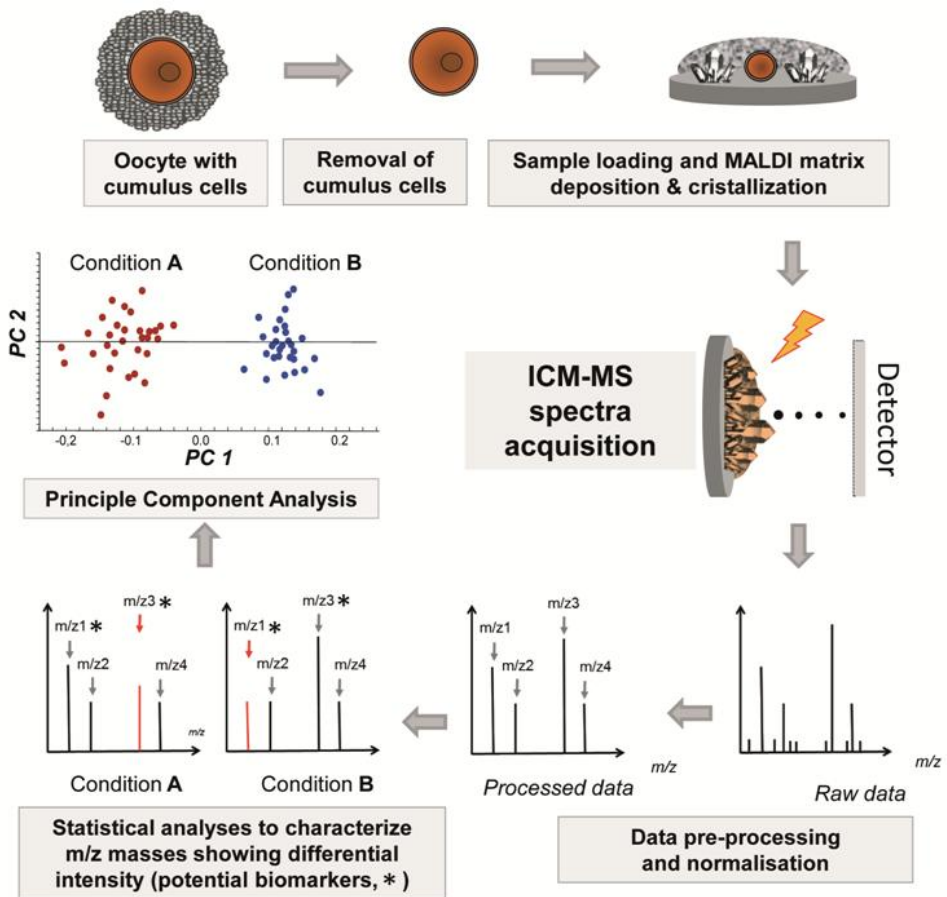
Oocyte transcriptomics. Global analysis of bovine oocyte transcriptome since 2003 [5] is now became a routine approach because of development of RNA amplification methods, new generation RNA sequencing and global annotation of bovine genome. Few or even single oocytes are sufficient to amplify RNA and perform transcriptomic analysis, so gene expression of more or less competent oocytes could be compared. For example, genes *SLC25A16*, *PPP1R14C*, *ROBO1*, *AMDHD1* and *MEAF6* were shown to be differentially expressed in the oocytes, obtained from the Monbeliarde animals with high or low oocyte potential to produce viable embryos after *in vitro* maturation (IVM) [6]. The comparison of more competent oocytes from the large follicles (> 8mm) with the oocytes with lower competence from the small follicles (< 3 mm) allowed the identification of several genes involved in crucial functions such as transcriptional regulation (*TAF2*), chromatin remodeling (*PPP1CB*), energy production (*SLC25A31*), as well as transport of key molecules within the cell (*NAGPA*, *CYHR1*, and *SLC3A12*) [7]. Oocyte which were denuded from their cumulus cells before IVM were less competent than those matured within oocyte-cumulus complex, and some differently abundant transcripts were detected by microarray hybridization, although the most differences between these groups of oocytes were found in their lipid composition [8].

However, the relevance of oocyte transcriptomics has limitations because full-grown oocytes are transcriptionally quiescent, and therefore the difference in

transcript abundance between immature and mature oocytes is mainly related to either RNA degradation or degree of polyadenylation at 3'UTR [9, 10]. Consequently, in the oocytes, there is mainly no correlation between transcript abundance and level of the corresponding proteins: we have confirmed this for the main actors of oocyte meiotic maturation, like Aurora kinases, cyclin B1, CDK1, c-MOS, and CPEB1 in bovine oocyte during IVM [11].

Oocyte proteomics. In bovine oocyte, maturation process is accompanied with protein neosynthesis and different post-translational modifications (PTM), as protein phosphorylation or acetylation. Proteomic changes that occur in the oocyte during maturation and that define the quality of *in vitro* matured oocytes are not enough known, and so there is not an accurate way of evaluating/monitoring how different IVM protocols can affect the process, at single oocyte level. Proteomics has been employed with this objective. Classical proteomic approaches using gel separation and tandem mass spectrometry (MS) after liquid chromatography (LC-MS/MS) require a high amount of cells – from several hundreds to thousands oocytes per condition. The reason for this is that despite the oocyte being the largest cell in the organism (about 120 μm in diameter in cattle), it contains relatively low intra-cellular protein content. In average, bovine oocyte contains about 100 ng total proteins of which approximately 15% is attributed to zona pellucida – a thick extracellular coat which consists mainly of glycoproteins. Several studies have reported functional proteomic approaches performed on pools of immature and mature bovine oocytes [12, 13]. Numerous proteins including putative markers of oocyte developmental competence including proteins needed for fertilization, reprogramming, embryo genome activation and first cleavages during early embryo development were identified. Very recently, new efficient sample preparation using paramagnetic bead technology allowed the identification of more than 400 proteins in single human oocyte [14]. However, this procedure is very complex, and is not convenient for high-throughput quantitative analysis on individual oocytes.

In our laboratory, we have adapted original approach, Intact Cells Matrix-Assisted Laser Desorption/Ionization time-of-flight Mass Spectrometry (ICM-MS), to analysis of single bovine oocytes (Fig.). Being initially developed for bacteria phenotyping [15], ICM-MS has the ability to detect the most intense native molecular ions present directly in the biological sample over a relatively wide mass range (typically 100- 25,000 Da), with a high sensitivity (from the picomolar to the femtomolar range) and a high tolerance for contaminants such as salts. In addition, ICM-MS allows detection of very small proteins and peptides which are often lack when using classic LC-MS/MS methods. The method included only a few and simple preparatory including stripping oocytes from cumulus cells and washing them to eliminate any somatic cells and salt excess. The plating procedure is simple: oocyte was loaded onto a MALDI plate, overlaid with an adequate matrix and allowed to dry. Analysis was here performed using UltrafleXtreme MALDI-TOF/TOF instrument (Bruker) but other MALDI-TOF spectrometer may be also used. Using adequate pre-processing and bioinformatic analysis tools, ICM-MS profiles showed characteristic spectral features according to their real maturation stage or quality, and thus differential analysis is possible between different conditions. Consequently, ICM-MS constitutes a powerful tool for the direct analysis of complex peptide/protein mixtures within biological specimens such as crude extracts and intact cells and identify differential molecules, as it was shown for analysis on boar spermatozoa isolated from four different epididymal regions (immature to mature stage) [16]. As ICM-MS is based on the comparison of peptide and small proteins profiles rather than the comparison of single biomarkers, it is theoretically more prone to



Typical ICM-MS workflow. Individual oocytes are washed, then loaded to MALDI plate and recovered with the appropriate matrix. Lipid or peptide/protein spectra are obtained by MALDI-TOF mass spectrometer. Spectra are processed and aligned, and then total spectra intensity and peak heights are measured. Potential markers are discovered by statistical analysis of the spectra between different conditions. Principal component analysis is performed to discriminate the oocytes in different conditions.

identify phenotypic differences associated with a certain physiological, pathological or experimental condition, notably with a subfertility versus high fertility as shown for chicken semen [17]. ICM-MS is in fact, the possibility of using whole, untreated cells (i.e. no extraction or pre-fractionation), high throughput, ease of operation, simple sample preparation and the existence of established data-processing platforms and software for processing and quantification of spectral peaks (normalized peak height values) makes this technique particularly promising for applications in reproductive biotechnologies for analysis of different cells of reproductive organs.

To discover the possible factors of oocyte quality, we compared individual oocytes with different developmental competence using ICM-MS technology. Reproducible proteins/peptides fingerprints gathering a more than two hundreds of peaks were obtained for single oocytes, both immature and after IVM[18]. Immature and mature oocytes were easily discriminated by Principle Component Analysis using a number of peaks which varied significantly between the correspondent groups. Moreover, peptide/protein fingerprints obtained by ICM-MS allowed discrimination of the oocytes at the same maturation stage but with different developmental potential after IVF; for example, *in vivo* versus *in vitro* mature oocytes or oocytes after IVM, matured either with or without cu-

mulus cells, or oocytes from prepubertal calves versus adult cows.

ICM-MS analyses should be completed with the identification of the peaks present in the spectra according to their specific m/z (mass to charge ratio) values. Peak masses correspond to native, endogenous molecules present in the sample. Of all the MS-based identification approaches currently available, top-down (TD) proteomics by high resolution nano-LC-MS/MS constitute the best approach to identify endogenous peptidofoms and proteofoms, as it involves measurement of an intact molecular species and direct fragmentation, thus providing with a complete description of the primary structure of the protein and of its modifications [19]. Pool of oocyte-cumulus complexes and follicular cells was used to extract total proteins which were then employed for TD identification of the peaks which were observed in oocyte ICM-MS spectra. TD approach allowed identification of more than 350 of unique proteins which were represented by either intact small size proteins (environ 15%) or N-terminal, C-terminal or intermediate fragments of the proteins higher molecular weights. These fragments were shown to be products of proteolytic activities of different endopeptidases targeting specific sites or substrates. Numerous PTMs were also evidenced on these proteofoms. Among the identified markers of oocyte maturation, we found several known proteins involved in cytoskeleton organization (alfa- and beta-thymosin), chromosome organization (histones) or protein degradation (ubiquitin) [20].

Oocyte lipidomics. Intracellular lipids have several roles including structural functions while they are components of membranes, energy storage and molecular signaling. The importance of oocyte lipid metabolism during oocyte maturation for its developmental competence is now formerly recognized [21]. Active lipolysis, fatty acid synthesis and oxidation permanently occur in bovine oocyte and surrounding cumulus cells and increased during IVM [22]. Bovine oocyte is rich in lipid droplets inclusion, and during IVM the total lipid quantity decreased [8]. Different methodologies of lipid analysis were applied to the oocytes in different species and allowed characterize different lipid classes of oocyte content. Analysis of complex lipid species in single bovine oocyte has been reported using MALDI MS, and some species of sphingomyelins, phosphatidylcholines and triacylglycerols were detected [23].

We adapted ICM-MS to establish lipid fingerprints from single bovine oocytes. The analytic procedure was similar to peptide/protein profiling except specific matrix and parameters of spectra acquisition. Lipids were easily detectable by ICM-MS, presented as numerous peaks of different intensity. By measurement of normalized peak height of all detected ions we compared lipid fingerprints of immature oocytes and oocytes after IVM and demonstrated significant increase in abundance of several peaks, identified as phosphatidylcholines and sphingomyelins, whereas abundance of two lower weight species (possibly free fatty acids) was significantly decreased [24].

Limitations of “omics” analysis of single oocytes. Technical hurdles of “omics” analysis of single oocyte caused by the minimal amount of biological material are evident and require a miniaturization of several methodologies for extracting and measurement of biological material. It seems being of less importance for transcriptomics because of the possibility of RNA amplification; however this step introduces the biases to real representability of the specific RNAs due to differential enrichment rate depending on either the difference in expression level or to transcript length. Also, the question of data normalization is recurrent in the analyses of the oocytes at different maturation stages.

Correct preservation of the samples before analysis is crucial. Whether oocyte RNA is enough protected from the ribonucleases by zona pellucida and,

in addition, RNA protectors are also available, oocyte lipids and proteins require reinforced more protection even stored frozen. Indeed, lipidomics must be performed as soon as possible after collection because of massive oxidation and degrading of lipids under atmospheric oxygen, and lipid profiles significantly change during storage at -80°C . Proteins seem to be more stable however ICM-MS performed with fresh oocytes generates much more informative and reproducible spectra than with frozen ones.

Whether an oocyte is used for molecular analysis, its capacity to develop to embryo could not be directly assessed. Oocyte quality therefore could be evaluated by the measurement of developmental competence of the oocytes matured in similar conditions or from the same animal as those which were analyzed. However, the variability between the oocytes recovered from the same animal and /or from the follicle of the similar size is very important. In fact, the quality of the oocyte at the start of the maturation process is thought as the key factor determining the proportion of oocytes developing to the blastocyst after IVM [25]. Therefore, this variability of individual oocytes must be considered before analysis by providing for sufficient number of biological replicates.

Use of cumulus cells to search non-invasive biomarkers of oocyte quality. Cumulus cells are physically and metabolically coupled with the enclosed oocyte, and due to permanent exchanges of small molecules (ions, metabolites, amino and fatty acids, AMPc etc), cumulus may reflect oocyte physiological status. Therefore, whether a part of cumulus cells are taken for analysis, the oocyte may be kept for fertilization and embryo development. A system capable of supporting the IVM, IVF and development of immature bovine oocytes to the blastocyst stage in an individually identifiable manner was reported [26]. More abundant in biological material, cumulus cells from individual oocytes could be used for transcriptomic analysis and gene expression data were correlated with embryo development of correspondent oocytes and thus allowed identification of the markers associated with oocyte quality [27]. Among these genes, 1-acylglycerol-3-phosphate O-acyltransferase 9, *AGPAT9*, involved in lipid metabolism, was over-expressed in cumulus cells of the oocytes stopped at 2-8 cells.

In our laboratory, we adapted ICM-MS profiling for analysis of both lipids and proteins in cumulus cells, surrounding individual oocyte by using either all detached cells or cumulus biopsies. ICM-MS of lipids in cumulus cells allowed clear discrimination of the profiles according to maturation stage or metabolic status of the correspondent oocytes [22]; ICM-MS protein/peptides signatures of cumulus cells were also specific and grouped in accordance with oocyte conditions.

By using the TD approach earlier described, numerous peaks from cumulus cell protein and lipid profiles were identified, and they constitute now the database for further research.

Thus, different “omics” technologies became available tools for analysis of individual oocytes and somatic follicular cells. Oocyte transcriptomics, proteomics and lipidomics on both oocyte and surrounding cumulus cells generate data in order to establish the accurate, fast and affordable tests that can help in the assessment of oocyte quality in assisted reproduction biotechnologies of farm animals. The combination of original mass spectrometry approaches, ICM-MS and TD, proved to be a suitable strategy to identify markers of oocyte quality in bovine using limited biological samples.

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