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Mitochondria and mitochondrial DNA in porcine oocytes and cumulus cells — A search for developmental competence marker

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ABSTRACT

The development of mammalian oocytes is dependent on bidirectional signaling with the surrounding cumulus cells. Among the numerous factors that contribute to oocyte developmental competence, the mitochondria and the mitochondrial DNA play pivotal roles. Although these highly abundant organelles have been well-studied in oocytes, their roles, abundance and metabolism remain elusive in cumulus cells. Therefore, the aim of our study was to analyze the correlation between the mtDNA copy number in cumulus cells and oocytes, as well as the mitochondrial distribution patterns in oocytes, using two groups of animals that differ in terms of the developmental competence of their oocytes. We determined a positive correlation between the mtDNA copy number in the cumulus cells and mtDNA copy number in oocytes of prepubertal pigs and negative correlation in cyclic gilts. These opposing correlations may reflect the differences in the developmental competence of the prepubertal and cyclic oocytes. We also hypothesize that observed differences may reflect different metabolism and energy requirements of the cumulus–oocyte complexes from prepubertal and cyclic gilts. The mitochondrial distribution patterns in the prepubertal and cyclic gilts were not different.

1. Introduction

The developmental competence of the oocyte is most often understood as the ability to resume meiosis, cleave after fertilization, implant and develop to term (Hunter, 2000). With the exception of the first characteristic, it would seem that the development of the embryo and its quality are completely separate subjects, but it is the quality of the oocytes that determines the chances that an embryo will develop properly. Therefore, in assisted reproduction techniques (ART), the crucial element is the starting material, and thus the history of the donor (Krisher, 2013). Age, puberty, health and nutrition are well-known factors that directly or indirectly shape an oocyte's developmental potential through interaction with its growth environment in the follicular fluid and the surrounding cumulus cells (Dumesic et al., 2015). For years, both the fluid and the cumulus cells have been the subjects of intensive research to identify non-invasive markers for the assessment of oocyte quality on animal models and human medicine. Cumulus cells have primarily been tested to analyze gene expression levels, whereas the follicular fluid was tested for the presence of metabolites, fatty acids, microRNAs and proteins (Dias et al., 2014; Khan et al., 2013; Matoba et al., 2014; Sinclair and Garnsworthy, 2010; Sutton et al., 2003). During follicle development, from primordial follicle to the time of ovulation, the oocyte and surrounding cumulus cells interact extensively through paracrine signaling, which may involve a bidirectional functional connection between growth factors and receptors. The cross-talk between oocyte and cumulus cells is essential for metabolism, oocyte meiotic arrest and resumption. On the other hand oocyte paracrine factors like members of transforming growth Beta factor family (GDF9, BMP15) control and regulate the cumulus growth and expansion. Recent studies show that cumulus cells play a preventive role against inhibitory effect of high lipid concentration in follicular fluid environment (Aardema et al., 2013). However, few studies have described mtDNA analyses in cumulus cells. This is in contrast to studies of oocytes from many species, in which the mtDNA copy number and mtDNA mutations have been widely described. Nevertheless, the results of the mtDNA analysis on the oocytes and embryos from pigs, cattle, mice and humans indicated that the mitochondria and mitochondrial DNA have key roles in shaping developmental competence (Brevini et al., 2005; El Shourbagy et al., 2006; Reynier et al., 2001; Spikings et al., 2006; Van Blerkom, 2007, 2008, 2011). It was shown that only oocytes with at least 100,000 copies of mtDNA are capable of being fertilized and developing into preimplantation stage embryos (May-Panloup et al., 2007; Spikings et al., 2006, 2007). This is dictated by the fact that mtDNA replication occurs in the growing oocyte and preimplantation blastocyst, while the mitochondria and mtDNA pool are diluted with each cell division between these stages (Cree et al., 2008; Spikings et al., 2007; Wai et al., 2010). Consequently, oocytes exhibiting poor morphology or poor developmental competence will be characterized by a significantly lower mtDNA number, leading to developmental failure. While the mtDNA in oocytes has been extensively examined, cumulus cells have not yet been the subject of such analyses.

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Another feature of the mitochondria in oocytes is their redistribution from the peripheral parts of cytoplasm to the center during growth and maturation, possibly reflecting the local energy demands during different stages of development (Stojkovic et al., 2001; Sun et al., 2001). This mechanism is observed to a lesser extent in oocytes matured in vitro compared to those matured in vivo; however, the mitochondrial distribution may be used as a marker of oocyte cytoplasmic maturation in different experimental groups (Brevini et al., 2005). Therefore, based on our previous studies on porcine oocytes and observations of mitochondrial staining, we decided to correlate the mtDNA copy number in cumulus cells and oocytes to determine whether it is a potential non-invasive marker of the oocytes selected for assisted reproductive techniques. In our experiment, we used oocytes collected from prepubertal and cyclic gilts to create two groups that differ in their developmental competence (Bagg et al., 2004, 2005, 2007; Pawlak et al., 2011, 2012). Our recent results showed that different oocyte growth environments (follicular fluids) may cause the observed differences in the competence of prepubertal and cyclic oocytes. Here we hypothesized that not only the previously reported altered gene expression in cumulus cells might have an impact, but also altered mtDNA content.

2. Materials and methods

Unless stated otherwise, all chemicals and reagents used in this study were purchased from Sigma-Aldrich (Germany). The ovaries utilized in our experiment were collected post-mortem from commercially slaughtered gilts.

2.1. Recovery of the cumulus–oocyte complexes (COCs)

The cumulus–oocyte complexes (COC) were collected from the ovaries of commercially slaughtered gilts at the age of 5–6 months and weights of 100–110 kg. The ovaries were excised and placed in a separate plastic container for transport. The ovaries were transported to the laboratory in a thermally isolated flask within 2 h. Upon arrival, the ovaries were divided into 2 groups based on their morphology: prepubertal (P, lack of corpus luteus, several 1–4 mm follicles) and cyclic (C, the presence of corpus luteus or albicans, 3–6 mm follicles). The COCs were aspirated from the 2–5 mm follicles with a syringe, placed in Hepes-TALP medium and morphologically evaluated under a stereomicroscope. Only the cumulus–oocyte complexes with evenly granulated cytoplasm and at least three compact cumulus cell layers were selected for the experiment (Fig. 1). Half of the collected COCs were analyzed directly after the collection, whereas the other half were subjected to in vitro maturation and then analyzed. Consequently, four groups of porcine COCs were generated: 1) P (without IVM), 2) P IVM, 3) C (without IVM) and 4) C IVM.

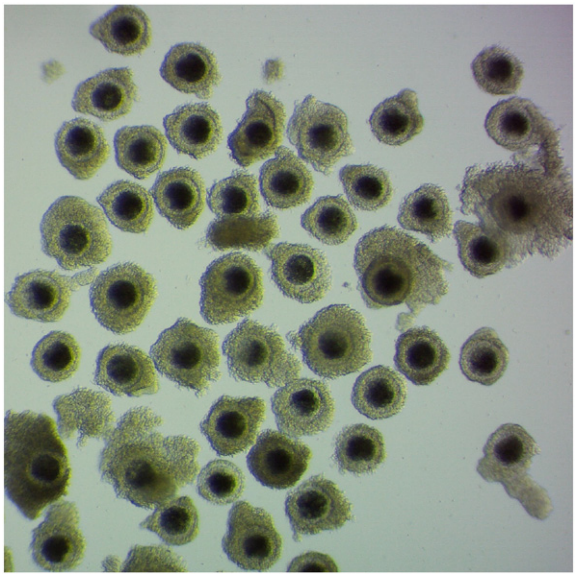

Fig. 1. Cumulus oocyte complexes — COCs exhibiting good morphology and used for in vitro maturation.

Each of the investigated parameters was analyzed using three replicates (three independent pools of oocytes) before IVM and three replicates after in vitro maturation. The COCs were collected from batches of P and C ovaries and randomly allocated into the specific experimental groups after morphological evaluation.

2.2. In vitro maturation (IVM)

IVM was performed in basic NCSU23 medium supplemented with 10% (v/v) porcine follicular fluid, 10 U/ml hCG (Folligon, Intervet, Poland), 10 U/ml PMSG (Choluron, Intervet), 0.1 mg/ml cysteine and 50 μg/μl gentamicin sulfate, as previously described. Briefly, the maturation protocol involved two steps: 1) an initial 20 h culture in hormone-supplemented NCSU23 medium and 2) a 24 h incubation in freshly made, hormone-free NCSU23 medium. The incubation was performed in 500 μl IVM medium in four-well plates (NUNC 176740) at 39 °C in a 5% CO_2 humidified atmosphere. The expansion and mucification of the cells are extremely intense in the porcine in vitro maturation system, such that it is impossible to isolate the cumulus cells from a single oocyte that was matured in a group in a single well. Therefore, we used a system that would enable us to easily separate the cumulus cells from each oocyte after maturation. Each well consisted of 14 small wells to promote the maturation of individual cumulus oocyte-complexes (Suppl. Fig. 1). The COCs remained in individual wells throughout maturation to ensure the effective isolation of additional cumulus cells without contamination from the neighboring COCs.

The follicular fluid (FF) was prepared for IVM by aspirating it from the ovarian follicles (3–5 mm) and transferring to conical tubes. The FF was centrifuged at 10,000 rpm for 1 min, and aliquots of the supernatant were frozen in liquid nitrogen in Eppendorf tubes (Eppendorf, Germany). The follicular fluid was stored for one month and used to supplement the IVM medium. The follicular fluid that had been collected from prepubertal and cyclic gilts was used for IVM supplementation of the prepubertal and cyclic oocytes, respectively.

The maturation efficiency (the number of MII oocytes was divided by the number of all analyzed oocytes) was estimated by DAPI staining and visualization under a fluorescent inverted microscope (Zeiss Axiovert 200, Germany). The oocytes at the MII stage were characterized by the presence of an oocyte metaphase plate, accompanied by the chromatin of the first polar body. For this purpose, the denuded oocytes were fixed in 4% paraformaldehyde for 30 min at room temperature and placed on slides under coverslips in a 0.75 μg/ml DAPI solution (Vector Laboratories).

2.3. Mitochondrial DNA (mtDNA)

2.3.1. Sample preparation and DNA extraction

Before analysis, all cumulus cells surrounding the oocytes were removed by vigorous pipetting with a micropipette. The denuded oocytes were washed 3× in a 0.2% PBS/PVP solution to ensure that the samples were not contaminated with cumulus cells. The individual oocytes and cumulus cells were placed in separate 1.5 ml LoBind tubes (Eppendorf) with 200 μl of PBS and immediately frozen in liquid nitrogen. All samples were stored at −80 °C. The total DNA was extracted with the High Pure PCR Template Preparation Kit (Roche, Switzerland) according to the manufacturer's protocol. Briefly, the samples were incubated in binding buffer with proteinase K for 10 min at 70 °C. Afterwards, isopropanol was added, and the lysate was placed on a filter column and centrifuged at 8000 g for 1 min. The next steps involved addition of the inhibitor removal buffer, followed by 2× washing solutions. Finally, the total DNA was eluted into a fresh 1.5 ml LoBind tube with 200 μl