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In vitro production of bovine embryos: cumulus/granulosa cell gene expression patterns point to early atresia as beneficial for oocyte competence

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Abstract

In vitro production (IVP) of bovine embryos has become widespread technology implemented in cattle breeding and production. Here, we review novel data on cumulus/granulosa cell gene expression, as determined by RNAseq on cellular material from pooled follicular fluids at the single animal level, and relate these finding to previous data on oocyte developmental competence and ultrastructure. The cumulus/granulosa cell gene expression patterns indicate that early follicular atresia is associated with increased blastocyst yield and this hypothesis is supported by previous data on oocyte competence and ultrastructure.

Keywords: IVP, biomarkers, transcriptomics, granulosa cells, atresia, cattle, oocyte competence.

Introduction

In the Western World, there are increasing governmental and public demands for environmentally friendly dairy products derived under conditions securing animal health and welfare. These demands are reflected in innovation programs and proposed laws specifying production methods, but also in the increasing demands from e.g. Danish consumers for organic products resulting in a 60% higher price for the primary organic milk.

Breeding can significantly improve feed-conversion efficiency and resilience and lower methane-emission in cattle (Colditz and Hine, 2016; Negussie *et al*., 2017). Since the beginning of 2000, novel technologies have made it possible to accelerate the rates of genetic gain in domestic cattle by genomic selection of calves (Blasco and Toro, 2014; Daetwyler *et al*., 2014). The association between phenotypic traits and genomic markers are getting more and more reliable and includes increasing focus on resilience and feed-efficiency. Denmark is one of the leading countries with regards to correlation of phenotypes for production and health to genomic markers according to the Nordic total merit (NTM) in the form of single nucleotide polymorphisms (SNPs) using the Illumina BeadChip. Likewise, Denmark and other countries put great emphasis in registration of methane-emission in dairy cows with the prospect of identifying genomic markers for low emission (Sousa *et al*., 2017).

Ultrasound-guided ovum pick-up (OPU) and *in vitro* production (IVP) of embryos allows for a significant improved utilization of the female gene pool as multiple embryos of a specific gender can be produced from elite females. If these technologies are used on very young heifers and combined with genomic selection of the embryos, performed on a small cell biopsy before the transfer to recipients, the gain of a significant shortening of the generation interval is added (Kasinathan *et al*., 2015). The combined application of OPU, IVP and genomic selection has until recently posed technical challenges with respect to embryo handling and DNA amplification. However, great progress has been achieved by e.g. the EmbryoGene consortium in Canada with respect to optimization of methodologies, and the combination of OPU, IVP and genomic selection of embryos is believed to hold great promises in cattle breeding (Saadi *et al*., 2014).

At present, there is no commercial use of OPU/IVP in Denmark, and the application of the technologies for scientific purposes has been on hold until 2014. These restrictions have been due to the fact that bovine IVP has, over time, been hampered by impaired embryo quality resulting in the large offspring syndrome (LOS; Behboodi *et al*., 1995; Kruip and Den Daas, 1997; Van Wagtendonk-de Leeuw *et al*., 2000). Over the past years, improved formulations of the media for oocyte maturation and embryo culture have resulted in improved embryo quality, even though increased early embryo loss may still be seen over the first trimester of pregnancy (Alberto *et al*., 2013). Aberrant epigenetic programming, with respect to e.g. DNA methylation, is a potential factor underlying these losses (Hori *et al*., 2010; Chen *et al*., 2015). Nevertheless, IVP has become widely accepted for commercial production of bovine embryos in South and North America and parts of Europe with Denmark lacking behind.

Great advances have been done with respect to the development of media for bovine IVP, which to a great extent has eliminated LOS. However, less attention has been paid to the variable yield of competent oocytes between donor cows (Tamassia *et al*., 2003) as well as to the fact that the basal efficiency of IVP is still relatively low with only 35-45 % of

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cumulus-oocyte complexes (COCs) of good morphology resulting in blastocysts (Mayes and Sirard, 2001; Sirard *et al*., 2006; Muñoz *et al*., 2014).

Transcriptomics can help in identifying biomarkers for oocyte competence (Uyar *et al*., 2013). In cattle, many studies have exploited the power of Next-Generation Sequencing (NGS) technologies to identify follicular biomarkers (Orozco-Lucero and Sirard, 2014). Cumulus and granulosa cells are intimately coupled to the oocyte through paracrine and intercellular communication systems and play major roles in the development of oocyte competence (Macaulay *et al*., 2015). Hence, these cellular compartments may reflect the quality of the oocyte and represent assessable targets for analyses, as they are aspirated together with the COCs.

Previous studies have dissected how the granulosa cell profile varies between follicles with different characteristics, for example between follicles at different developmental stages (Girard *et al*., 2015), between follicles of different sizes (Hatzirodos *et al*., 2014b) and between healthy and atretic follicles (Hatzirodos *et al*., 2014a). None of these studies, however, have been conducted at the single animal level to give information on the particular animal's quality as a potential oocyte donor. The aim of the present manuscript is to review our recent attempt to analyse the transcriptome of the collective cumulus/granulosa cell transcriptome of individual oocyte donor cows in order to dissect potential gene expression patterns associated with high competence of the donor for IVP (Mazzoni *et al*., 2017). These data are subsequently combined with previous data from our group on oocyte ultrastructure during follicular dominance and atresia in order to propose a mechanistic understanding of oocyte IVP competence (for review, see (Hyttel *et al*., 1997).

Expression of candidate genes associated with IVP outcome at the single cow level

In order to find associations between the collective cumulus/granulosa cell transcriptome and IVP competences within individual donor cows, COCs and follicular fluids were collected from 67 individual cows and processed for IVP including *in vitro* maturation, fertilization and culture (Mazzoni *et al*., 2017; Fig. 1). On day eight after fertilization, embryos from all animals were scored with regard to three parameters: the blastocyst rate was computed for each animal as the number of blastocysts over the total number of inseminated COCs; a kinetic score was obtained by visual classification of each blastocyst as non-expanded, expanded or hatching/hatched; and a morphology score was obtained by visual classification of each blastocyst as poor, good or excellent.

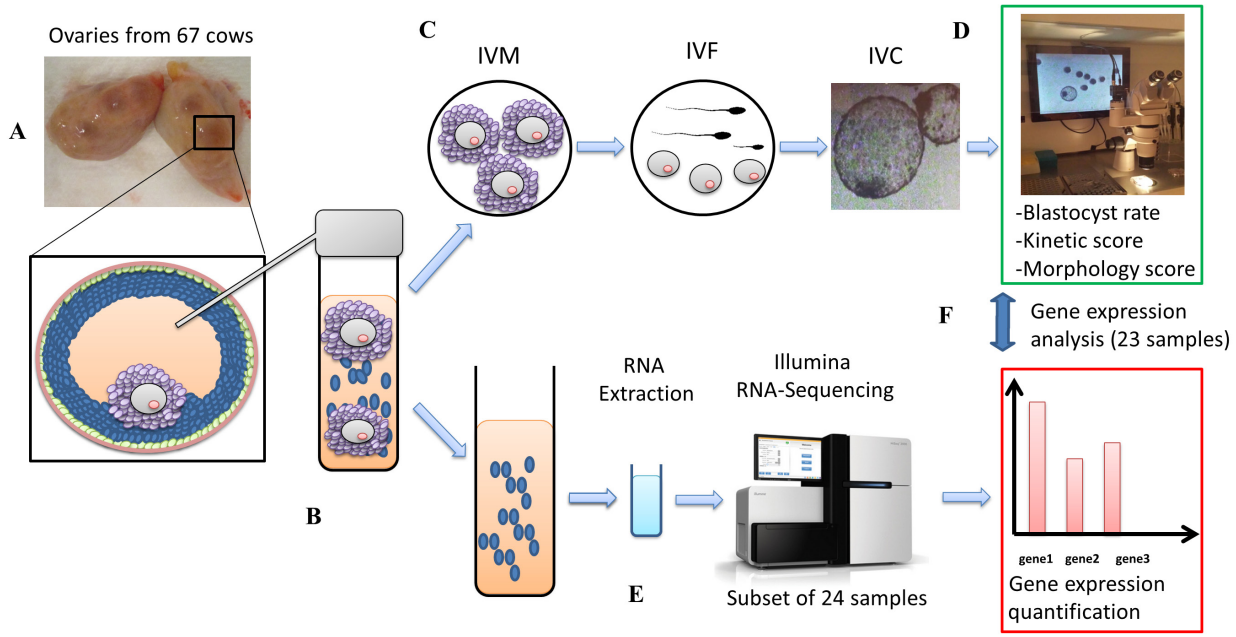

Figure 1. Experimental set-up used in Mazzoni *et al*. (2017). A) Ovaries from 67 Danish cows were collected after slaughter and each pair of ovaries kept separate. B) The COCs from each animal were aspirated with a vacuum pump and kept separate. C) The COCs were processed for *in vitro* production (IVP) including *in vitro* maturation (IVM), insemination (IVF) and culture (IVC) until the blastocyst (BL) stage (day 8). D) IVP parameters for each animal (BL rate, kinetics and morphology) were evaluated at day 8. E) RNA was extracted from the follicular cells contained in the aspirated fluids. A subset of 24 samples was selected and sequenced; F) RNA sequencing analysis was performed to identify genes associated with IVP performances.

Centrifuged cell pellets (cumulus/granulosa cells) from the follicular fluids were processed for RNA sequencing and bioinformatic analyses (Mazzoni *et al*., 2017). For the RNA analysis, only Holstein first- or multiple-lactation cows were used and the 24 samples with higher RNA quality (RNA integrity number) were

selected. One sample was excluded during the quality control procedure. Consequently, the final association between gene expression patterns and IVP outcome was performed on a selected group of 23 cows.

As referenced above the cumulus/granulosa transcriptome is associated with follicular status. COCs aspirated for IVP originate from follicles of a very heterogeneous background and will include dominant and subordinate follicles on ascending and descending slopes of the follicular waves (Forde *et al*., 2011). Interestingly, we found that two of the most important genes related to follicular development, i.e. the FSH receptor gene and the P450 aromatase gene, were expressed on average constantly across all the animals. This fact indicated that there were no systematic differences in follicular status between the animals. Hence, we consider genes that under these circumstances are correlated with IVP outcome as good candidate genes for understanding the molecular background for oocyte competence and potentially predicting the individual cows IVP outcome by identification of assessable biomarkers. The three IVP parameters were scored independently of each other. Hence, it is our hypothesis that genes significantly correlated with all three parameters are the most significant with respect to oocyte competence and potential biomarker discoveries.

The expression of seven genes was noted to be significantly associated with all three blastocyst parameters: Expression of STC1 and Mx1 were positively correlated, while expression of BEX2, RGN, HEY2, TXNDC11 and TNFAIP6 were negatively correlated with a good IVP outcome. Most of these genes have previously been found to be involved in the control of follicular development and oocyte developmental potential.

STC1 is highly expressed in both *in vivo* and *in vitro* matured oocytes (Mamo *et al*., 2011). The STC1 protein is secreted and exerts paracrine control of granulosa cell development, and it is expected to play a critical role in feedback loops between cumulus/granulosa and oocytes (Luo *et al*., 2004). A pro-apoptotic function of STC1 has previously been reported (Law *et al*., 2008; Guo *et al*., 2013).Thus, the increase of STC1 expression in cows with good IVP outcome could be associated with the presence of early atresia. Mx1 is involved in interferon signalling together with IRF and IFNAR, which were both identified as being correlated only to blastocyst rate, indicating that in particular Mx1, but also the other two genes, are of significance for oocyte competence in accordance with observations in other species including man (Lédée *et al*., 2008). Moreover, Mx1 has been reported as inducing cell death and apoptosis (Mibayashi *et al*., 2002). Hence, upregulation of Mx1 may consequently be associated with early atresia and, hence, improved IVP outcome (Fig. 2A).

BEX2 has previously been reported to be upregulated in large follicles as compared with their smaller counterparts (Hatzirodos *et al*., 2014b). BEX2 acts as an inhibitor of apoptosis in mitochondria and may, thus, prevent follicular atresia. Hence, downregulation of BEX2 may be associated with increased apoptosis and early atresia and, hence, improved IVP outcome (Fig. 2A). RGN has previously been found as highly expressed during follicular dominance and, interestingly, as acting to increase granulosa cell survival (Li *et al*., 2016). Again, downregulation of RGN may be associated with early atresia and, hence, improved IVP outcome (Fig. 2A). HEY2 encodes a transcriptional repressor, which is a downstream target of the Notch cell signalling. Interestingly, the expression of NOTCH2 was significantly correlated with low blastocyst rate although no significant correlations with kinetics and morphology was noted. It may be speculated that downregulation of HEY2 and NOTCH2, and of notch signalling as such, may induce apoptosis as it has been demonstrated in mice (Zhang *et al*., 2011). Again, downregulation of HEY2 may consequently be associated with early atresia and, hence, improved IVP outcome (Fig. 2A).

TXNDC11 encodes a protein with the thioredoxin domain that might act as a redox regulator. TXNDC11 expression has never been associated to oocyte competence in granulosa cells, although other thioredoxin proteins have been associated with the control of ovarian follicular atresia through scavenging action on reactive oxygen species (ROS; Townson and Combelles, 2012). ROS represent one of the major contributors to oxidative damage (Cadenas and Packer, 1999; Patel *et al*., 1999; Turrens, 2003; Townson and Combelles, 2012) and cell death (Ott *et al*., 2007). We speculate that a lower expression of TXNDC11 could lead to an increase in the concentration of ROS and consequently promote atresia. Again, downregulation of TXNDC11 may be associated with early atresia and, hence, improved IVP outcome (Fig. 2A).

The expression of TNFAIP6 in granulosa cells has been correlated with decreased bovine oocyte competence after ovarian stimulation (Gilbert *et al*., 2012). The TNFAIP6 protein is an important component of the extracellular matrix (ECM) thanks to its hyaluronal-binding LINK domain. The ECM promotes cell survival and proliferation of granulosa cells during the follicle development in cattle (Woodruff and Shea, 2007; Salilew-Wondim *et al*., 2014; Ploutarchou *et al*., 2015). Again, downregulation of TNFAIP6 may be associated with early atresia and, hence, improved IVP outcome (Fig. 2A).

An important consideration during the selection of candidate genes encoding potential biomarkers for a particular cow's competence for IVP is the subcellular localization of their protein products. Genes whose protein products are secreted into the extracellular space (including the follicular fluid and eventually blood plasma) can potentially be measured in these fluids and used as biomarkers of IVP traits. Among the candidate genes described above, only STC1 encodes a protein, which is secreted.

Gene expression patterns in relation to follicular size and atresia

The functional analysis of the seven candidate

genes pointed to their potential involvement in follicular atresia. Previously, gene expression in cattle has been reported in healthy *vs*. early atretic antral follicles (Hatzirodos *et al*., 2014a) and in small vs. medium and large follicles (Hatzirodos *et al*., 2014b). A comparative analysis revealed that 65% of the genes identified as differentially expressed in early atretic follicles vs. healthy follicles (Hatzirodos *et al*., 2014a) showed the same trend (being up- or downregulated) in our study and this percentage increased to 90% considering the top 25% of the genes positively correlated with good IVP outcome in our study (Fig. 2B). Conversely, 84% of the genes identified as differentially expressed in medium and large follicles *vs*. small follicles (Hatzirodos *et al*., 2014b) showed the opposite trend in our study and this percentage increased to 92% when considering the top 25% genes correlated to blastocyst rate in our study. Taken together, the gene expression patterns in our study combined with the data on atresia and follicle size indicates that good IVP outcome is positively correlated with early atresia and negatively correlated with follicle size. Hence, very interestingly, this relationship points to small early atretic follicles as yielding the most competent oocytes for IVP.

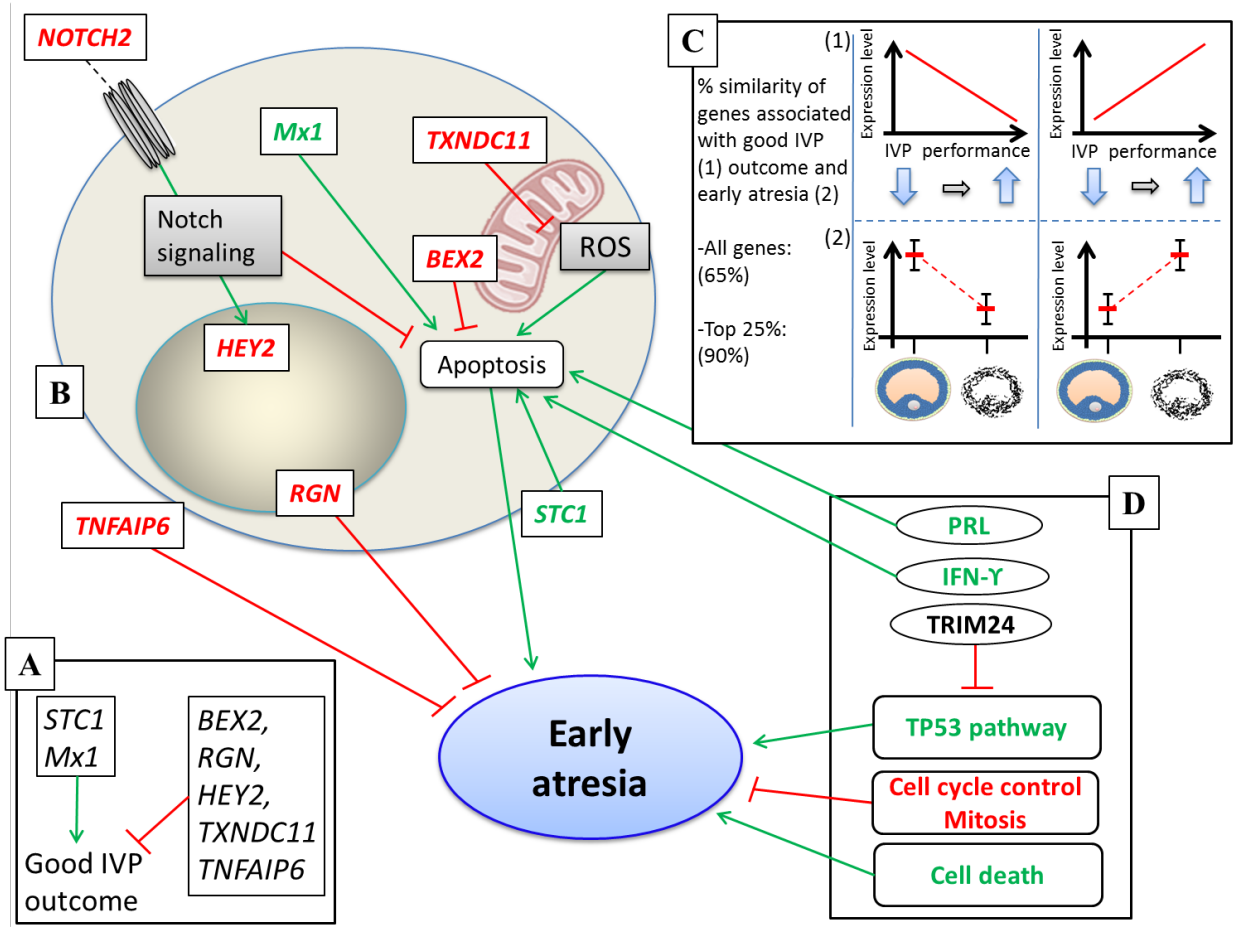

Figure 2. Summary of the evidences that lead to the hypothesis that early atresia is correlated with good IVP outcome. The figure summarizes all the bioinformatics evidences that lead to the atresia hypothesis. A) The expression level of 7 genes were significantly correlated with all parameters of a good IVP outcome: Blastocyst rate, kinetic score and morphology score. B) Schematic representation of a granulosa cell with the candidate genes and the mechanisms that support the positive correlation between early atresia and good IVP outcome. C) Representation of the comparison between the expression profiles in our study (1) and the expression profiles of atretic versus healthy follicles from Hatzirodos *et al*. (2014a; 2). D) Enriched pathways and upstream regulators associated with IVP outcome. Text color code: Green = positive correlation with good IVP outcome; red = negative correlation with good IVP outcome. Shape code: Rectangles = candidate genes; ovals = upstream-regulators; rounded rectangles = biological processes and pathways, grey-filled rectangles = molecules or molecular signalling. Arrow code: Green arrows = activation; red lines = inhibition.

Pathway enrichment and upstream regulator analysis associated with follicular atresia

Functional pathway enrichment analysis can identify biological functions and pathways overrepresented in the set of genes associated with a certain trait of interest. Functional analysis with Ingenuity® Pathway suite (IPA®) predicts the activation state (activated or inhibited) of the biological processes and of the main upstream regulators for the genes associated with a specific trait of interest. Functional enrichment was performed to extract biological insight

related to the IVP process from the gene expression profiles and provided new evidences sustaining the relationship between small early atretic follicles and good IVP outcome. Hence, several biological pathways or processes were identified as being of significance for the IVP outcome. In details, processes for the control of cell proliferation and development (mitosis, cell cycle control) were negatively correlated with good IVP outcome while cell death process and the TP53 pathway were positively correlated with good IVP outcome (Fig. 2C).

The upstream regulator analysis was performed to identify key proteins responsible for the control of the expression of the genes that we found associated with good IVP outcome. Therefore, even if the expression of the upstream regulators was not identified in our dataset as being absolutely differentially expressed they indirectly represent potential candidate proteins for IVP outcome. The important upstream regulators of genes associated with good IVP outcome were TRIM24, PRL and IFN-ϒ (Fig. 2C).

TRIM24 was identified as an upstream repressor of TP53 promoting the degradation of this protein. Hence, TRIM24 is thought to prevent TP53-induced apoptosis and, thus, atresia. The TP53 pathway is specifically interesting as it is upregulated at apoptosis (Fridman and Lowe, 2003) and has been identified as being activated when bovine growing follicles enter the plateau phase of the follicular wave and initiate atresia (Nivet *et al*., 2013). Hence, upregulation of the TP53 pathway may consequently be associated with early atresia and, hence, improved IVP outcome (Fig. 2C).

PRL or prolactin was predicted as being activated in cows with good IVP outcome by the upstream regulator analysis with IPA® The activation of the PRL pathway has previously been reported as positively correlated with oocyte competence (Nivet *et al*., 2013) and with the occurrence of atresia (Lebedeva *et al*., 1998). In the rat, PRL administration has on the one hand resulted in an increased number of atretic follicles *in vivo* (Besnard *et al*., 2001) and, on the other hand, in a decrease in the abundance of granulosa cells in late stages of cell death *in vitro* (Lebedeva *et al*., 1998; Heleil *et al*., 2010) combined with an increase in embryo development to the morula and blastocyst stages (Kuz'mina *et al*., 2001). Again, upregulation of the PRL pathway may consequently be associated with early atresia and, hence, improved IVP outcome (Fig. 2C).

The expression of IFN-γ was not observed in our granulosa cell samples. However, the upstream regulator analysis with IPA® predicted IFN-γ to be activated. Within the ovary, IFN-γ is only synthesized by immune cells (Best *et al*., 1995). The protein enhances apoptosis and it has been found exclusively in atretic follicles in human (Best *et al*., 1995; Best and Hill, 2000). Again, upregulation of the IFN-γ pathway may consequently be associated with early atresia and, hence, improved IVP outcome (Fig. 2C).

Interestingly, we found that activation of the immune system was negatively correlated with good IVP outcome. We speculate that immune system activation is related to late atresia whereas the early atresia has not yet activated this type of response. This is partially confirmed by previous studies in human, where immune cells and, in particular, macrophages are abundantly recruited within the follicles at an advanced stage of atresia (Petrovská *et al*., 1996; Takaya *et al*., 1997; Gaytan *et al*., 1998). Again, this speculation supports the notion that early atresia, but not late atresia, is positively correlated with good IVP outcome.

However, macrophages have also been found to be present in healthy follicles and their abundance increases during follicle growth (Wu *et al*., 2004). It has been suggested that macrophages promote granulosa proliferation (Fukumatsu *et al*., 1992) or atresia by regulating the balance between cellular proliferation and apoptosis through the secretion of factors like TNFα (Kaipia *et al*., 1996; Wu *et al*., 2004) or IFN-γ (Mazzoni *et al*., 2017) as previously described. These mechanisms are still debated and must be addressed in future studies.

Oocyte ultrastructure and early atresia

The theory that early atresia is associated with good IVP outcome has been addressed earlier (Moor and Trounson, 1977; Wurth and Kruip, 1992; Feng *et al*., 2007) and it has directly been demonstrated that embryo yield is positively correlated with early atresia whereas late atresia has a negative impact (De Wit *et al*., 2000). Accordingly, the developmental potential of oocytes has earlier been reported as being positively correlated with granulosa cell apoptosis, which is widely used to identify atretic follicles (Feng *et al*., 2007; Heleil *et al*., 2010).

Along this line, previous ultrastructural studies performed in our lab also point to potential underlying explanations of the positive correlation between good IVP outcome and early atresia. Hence, studies of the ultrastructure of oocytes from dominant follicles approaching ovulation has clearly demonstrated that initial cumulus cell expansion and gradual retraction of the cumulus cell processes, attached to the oocyte through the zona pellucida, are initiated even prior to the LH peak (Assey *et al*., 1994). These somatic cell modulations are associated with changes in the oocyte nucleus, i.e. the germinal vesicle, which develops undulations of the nuclear envelope, likewise prior to the LH peak. After the LH peak these processes culminate in resumption of meiosis and progress of cytoplasmic oocyte maturation over a 24 h period leading to ovulation. We also found that the above-described sequence of processes can be observed in oocytes in the subordinate follicles of the follicular wave, i.e. follicles representing early atresia. Hence, in early atretic follicles, the oocyte apparently undergoes processes that mimic those seen in the dominant follicle approaching ovulation. Seen in this light, it is not surprising that oocytes harvested from early atretic follicles may be better qualified for entering final maturation *in vitro* as they may be "primed" for the process, whereas oocytes from healthy growing follicles are totally locked in meiosis and may experience problems in an immediate resumption of this process

when placed at IVM. Interestingly, coasting has been demonstrated to increase IVP embryo yield (Nivet *et al*., 2013); an effect that is likely also to be based upon initiation of early atresia in the follicular pool.

One approach to deal with the problem that oocytes may not be "primed" for IVM directly upon aspiration has been to induce a temporary arrest of oocyte maturation (Lonergan *et al*., 2003; Donnay *et al*., 2004; Vigneron *et al*., 2004). Years later, this concept was launched again through a specially designed medium, referred to as simulated physiological oocyte maturation (Albuz *et al*., 2010). The results have been varying, and a modified second version is now being tested (Gilchrist *et al*., 2015), illustrating that a practical solution to such a complex challenge is not always so easy. A dissection of this phenomenon is presently being concluded in another branch of the Brazilian-Danish GIFT project (Razza *et al*., 2016).

Conclusions

Cumulus/granulosa cell gene expression patterns indicate that early atresia is associated with increased blastocyst yield and this hypothesis is supported by previous data on oocyte competence and ultrastructure.

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