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In vitro production of bovine embryos: revisiting oocyte development and application of systems biology

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Abstract

In vitro production (IVP) of bovine embryos has become a widespread technology implemented in cattle breeding and production. The implementation of genomic selection and systems biology adds great dimensions to the impact of bovine IVP. The physical procedures included in the IVP process can still be improved, and aspects related to the oocyte donor, oocyte maturation and the recipients are addressed in the following. Also, some of the future aspects of genomic selection and systems biology are addressed with particular focus on the Brazilian-Danish collaboration in the so-called GIFT-project.

Keywords: cattle, embryo, fertilization, genome, oocyte.

Introduction

Over the past years, *in vitro* production (IVP) of bovine embryos has become implemented in cattle breeding in many countries. Particularly South and North America are regions where the technology has gained great impact. From a biological perspective, IVP has circumvented some of the early drawbacks related to serum-rich embryo culture conditions resulting in the large offspring syndrome (LOS). From a breeding perspective, the implementation of ultrasound-guided ovum pickup (OPU), quantitative genomics and systems biology has opened new possibilities for selection of oocyte donors and embryos for improved breeding strategies.

In spite of the improvements of the *in vitro* procedures for mimicking oocyte maturation, fertilization and initial embryonic development, IVP still needs refinements. The present review aims at revisiting, in particular, oocyte development, to pin point aspects where the bovine IVP systems are still suboptimal as compared with *in vivo* development, as well as aspects related to oocyte donors differing in reproductive stage and age. Also, we aim at pointing towards implementation of systems biology in bovine IVP and giving some practical considerations on the implementation of that technology in Denmark; a country where cattle breeding has reached a high level of perfection, but the implementation of bovine IVP has been delayed due to ethical animal welfare considerations related to OPU and LOS.

Revisiting oocyte development

Oocyte development in postpubertal heifers and cows

The development of a competent oocyte to be present for fertilization in the oviduct can roughly be divided into three distinct phases. The basic cell structure (ultrastructure) and developmental competence of the oocyte is generated during the first phase (the oocyte growth phase), when oocyte growth accompanies follicular growth from the primordial to the small (2-3 mm) tertiary (antral) follicle. During the antral phase when follicles in a cohort reach a diameter of about 3-5 mm, one dominant follicle is selected, as opposed to a group of subordinate follicles (Fig. 1; Ginther *et al.*, 1989). The ultrastructure of the oocyte in the dominant follicle enters a second phase (oocyte prematuration or capacitation), where it is modified reflecting an increase in oocyte developmental competence. In the ovulatory follicle, i.e. the dominant follicle of the last follicular wave, the oocyte undergoes a third and last phase of ultrastructural changes (oocyte maturation) during an approximately 24 h period between the peak of the LH-surge and ovulation.

When oocytes are aspirated for IVP from follicles of 2-8 mm in diameter, they are harvested from a heterogeneous and not fully competent pool of follicles. Hence, they originate from antral follicles of non-ovulatory or ovulatory follicular waves and from early dominant or early subordinate follicles. Consequently, the oocytes have not completed the acquisition of developmental competence, which was projected in the dominant follicle (oocyte prematuation), and some of them may even have encountered the environment of initial atretic follicles.

In order to reveal the ultrastructural changes during the three phases of oocyte development, we carefully processed oocytes for transmission electron microscopy from preantral follicles (primordial, primary and secondary follicles; (Fair *et al*., 1997) as well as from antral follicles, with appreciation of their status as

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being dominant or subordinate (Assey *et al*., 1994), and from preovulatory follicles approaching ovulation (Hyttel *et al*., 1986). The exact definition of dominant *vs*. subordinate follicles was achieved by administration of prostaglandin-F2alpha at day 7 after ovulation. This treatment stimulates ovulation of the dominant follicle of the first follicular wave. This simple model allows for harvesting of oocytes from well-defined stages of follicular dominance and subordination. The ultrastructural changes associated with oocyte development over the three phases of follicular development are presented in Fig. 2.

From the data presented in Fig. 2, it is obvious that the oocyte during the growth and prematuration phases undergoes marked changes that are reflected in its developmental competence and which are significant for obtaining full oocyte competence after maturation *in vivo* as well as *in vitro*. According to functional studies of bovine oocytes at different stages of their development, it appears that the oocyte achieves its basic competence for completing meiotic maturation to meiotic metaphase II (MII) at a diameter of around 110 µm (i.e. during the oocyte growth phase at the time when transcription decreases), and at a diameter of about 120 µm it attains the competence for subsequent blastocyst development. Hence, in oocytes harvested from follicles less than 3 mm in diameter, the oocyte has not completed the growth phase and build up the basic requirement of mRNA and proteins to sustain development (Fair *et al*., 1995, 1996, 1997). During the subsequent prematuration phase, the oocyte exhibits further structural modulations (Fig. 2) and its developmental competence increases (Assey *et al*., 1994). Strikingly, the oocytes of the subordinate follicles, which are entering into the early phase of atresia, may display very similar changes as seen on the oocyte of the dominant follicle. It will under normal circumstances be oocytes from follicles undergoing prematuration or initial atresia, which will be aspirated for bovine embryo IVP.

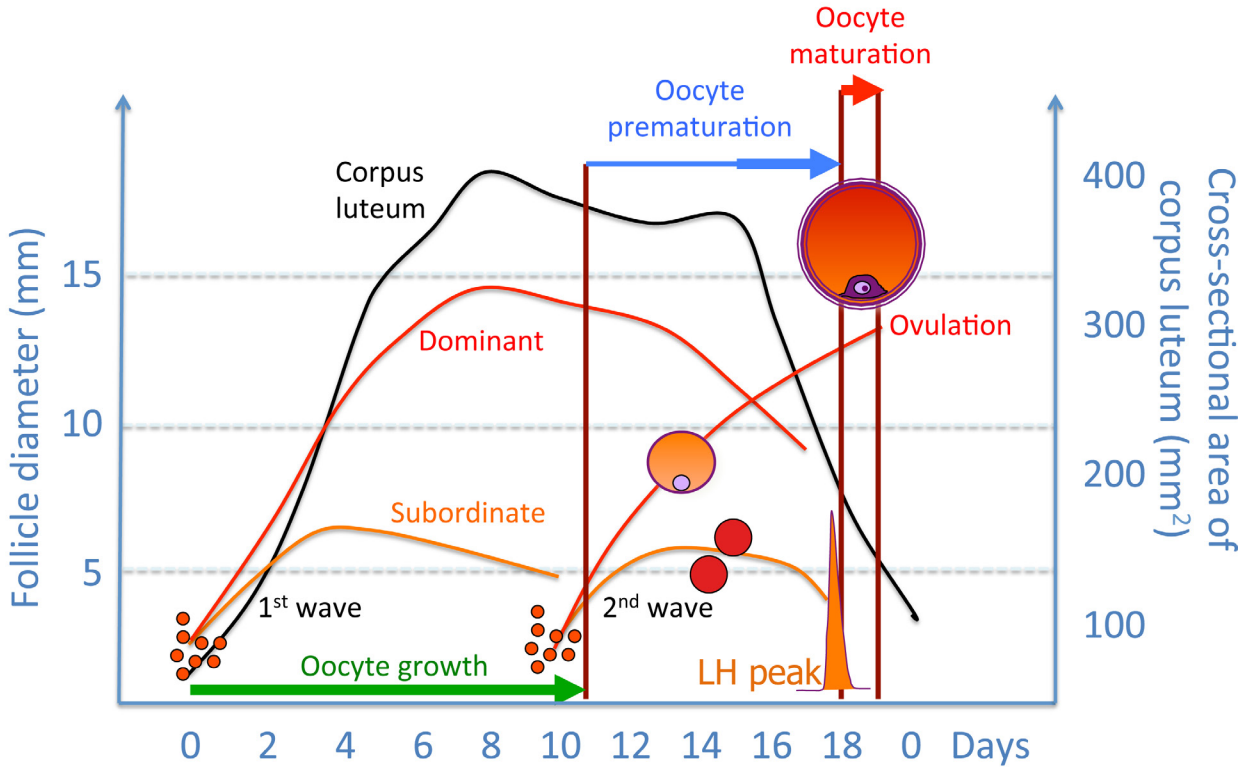

Figure 1. Follicular waves and phases of oocyte development in cattle (adapted from Ginther, 1998). The lower axis indicates days after ovulation.

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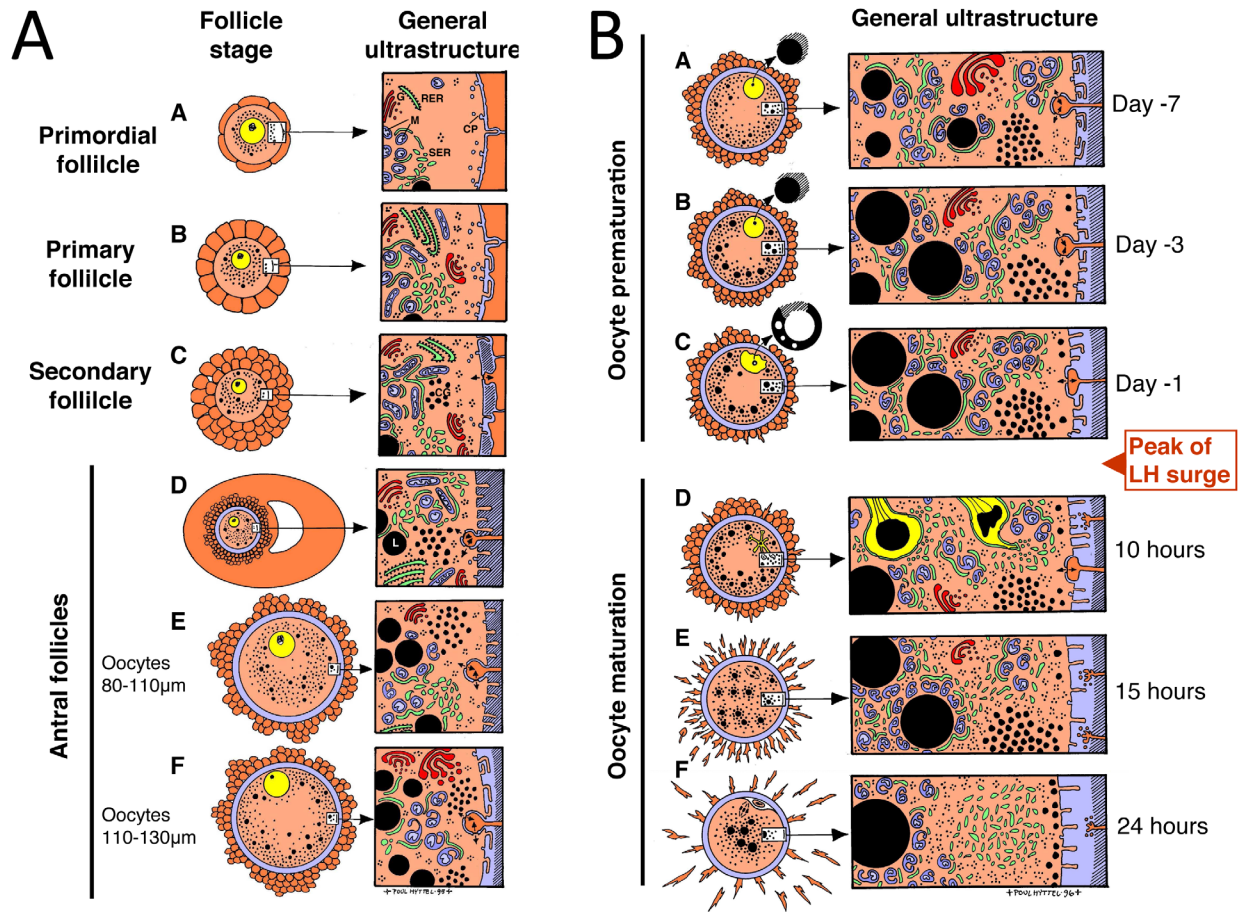

Figure 2. **(A)** Schematic drawing of bovine oocyte growth. **(A_A)** Primordial follicle with oocyte surrounded by a single layer of flattened granulosa cells. The central oocyte nucleus (yellow) is surrounded by round mitochondria (M), smooth (SER) and rough (RER) endoplasmic reticulum and small Golgi complexes (G). The oocyte cortex presents numerous coated pits (CP) and vesicles. The oocyte is transcriptionally quiescent. **(A_B)** Primary follicle with oocyte surrounded by a single layer of cuboidal granulosa cells. The eccentric oocyte nucleus is surrounded by round and elongated mitochondria. **(A_C)** Secondary follicle with oocyte surrounded by more than one layer of cuboidal granulosa cells. Small patches of zona pellucida material (hatched areas) have appeared and gap junctions (small arrows) developed between the oocyte and the granulosa cells. In the oocyte, the first small clusters of cortical granules (CG). The oocyte displays initial transcriptional activity. **(A_D)** Early tertiary follicle up to about 1mm. The follicular antrum has developed and the oocyte is surrounded by cumulus cells of which the innermost possess projections that penetrate the zona pellucida, invaginate the oolemma and make gap junctional contact to it. In the oocyte, the organelles have attained a more even distribution throughout the ooplasm, elongated mitochondria have become more numerous, lipid droplets (L) have become common, and the number and size of the cortical granule clusters have increased. Erect microvilli have become embedded within the zona pellucida. The oocyte is transcriptional active. **(A_E)** Tertiary follicle up to about 3 mm as represented by oocytes at 80 to 110 µm in diameter. The number of lipid droplets in the oocyte has increased. Oocytes less than 100 µm are transcriptionally active, whereas such at 100 to 110 µm transcription decreases in abundance. **(A_F)** Larger tertiary follicles as represented by oocytes at more than 110 µm in diameter. In the oocyte, the organelles have been dislocated to the periphery, the number of lipid droplets have increased as have the size of the Golgi complexes. The microvilli have been released from the zona pellucida and pile up in stacks in the perivitelline space. The peripheral oocyte nucleus presents has deceased its transcriptional activity to a minimum. **(B)** Schematic drawing of ultrastructural aspects of bovine oocyte prematuration in the dominant follicle up to the LH peak and maturation after the peak. **(B_A)** Oocyte from a dominant follicle 6 days before the LH peak. The general ultrastructure is identical with that obtained at the end of oocyte growth (A_F). **(B_B)** Oocyte from a dominant follicle 3 days before the LH peak. The number of microvilli stacks have decreased as have the size of the Golgi complexes, the amount of lipid droplets has increased, and the cortical granule clusters have dislocated to a more superficial location. **(B_C)** Oocyte from a dominant follicle on the day before the LH peak. Some individual corona cells display elongation and the corona cell projections have been retracted to a more superficial location, the perivitelline space has enlarged, the microvilli have become more erect, and the size of the Golgi complexes has been further reduced. Moreover, the envelope of the oocyte nucleus has become undulating and the nucleolar remnant has transformed into a ring-like structure. **(B_D)** Oocyte at "germinal vesicle breakdown" from an ovulatory follicle at 9-12 h after the LH peak. The perivitelline space develops further and in the oocyte the mitochondria tend to arrange around the lipid droplets and the nuclear envelope is dissolved into tubules of SER and microtubules appear adjacent to the condensing chromosomes. **(B_E)** Oocyte at MI from an ovulatory follicle at about 15 h after the LH peak. The number and size of the lipid droplets has increased and mitochondria have assembled around the droplets and these conglomerates have attained a more even distribution. Numerous ribosomes have appeared especially around the chromosomes and the size of the Golgi complexes has decreased further. **(B_F)** Oocyte at MII from an ovulatory follicle at about 24 h after the LH peak. The bulk of the cortical granules are distributed at solitary positions along the oolemma. The lipid droplets and mitochondria have attained a more central location in the ooplasm leaving a rather organelle free peripheral zone in which the most prominent features are large clusters of SER (adapted from Hyttel, 2011).

Age and status of the oocyte donor

There is an increasing trend within cattle breeding to apply OPU to younger and younger females and subsequently IVP in order to accelerate the genetic progress by reducing the generation interval. Also, some donors are in high lactation and/or in early pregnancy. Each of these different situations presents a particular challenge with respect to oocyte developmental competence, which can change according to status of the donor.

Ovaries in young females contain preantral and antral follicles, with the total number of follicles varying between individuals (Silva-Santos *et al*., 2013). The number of growing follicles rises rapidly between 50-80 days postnatally and increases up to 120 days. This follicle growth is stimulated by a transient increase in FSH and LH secretion, which later decreases until immediately prior to the first ovulation, at which time LH serum concentration and pulsatile secretory profile increase and change. During the prepubertal period, follicular waves are present and are preceded by FSH peaks (Erickson, 1966; Rawlings *et al*., 2003). Even though it is possible to aspirate antral follicles from very young heifers, the oocytes will have a decreased developmental competence compared to adults (Steeves *et al*., 1999), illustrated by differences in e.g. ultrastructure (Duby *et al*., 1996), oocyte metabolism (Steeves and Gardner, 1999), and cytoplasmic maturation (Salamone *et al*., 2001).

In high-yielding postpartum cows, low concentrations of circulating steroids have been measured, which could indicate that the dominant follicles are producing less oestrogen affecting the follicle growth phase (Lopez *et al*., 2004; Sartori *et al*., 2004). Furthermore, the negative energy balance in postpartum dairy cows has an adverse effect on oocyte quality, due to the changed endocrine and metabolic profiles (Leroy *et al*., 2008). In pregnant animals, follicular growth is possibly affected by elevated levels of progesterone (Adams *et al*., 1992; Dominguez, 1995).

As illustrated above, the expectations and results from OPU and IVP in cattle depend on the reproductive and physiological status of the oocyte donor, determined by factors such as genetics, age, breed, nutrition, pregnancy, milk yield etc. More research is needed to investigate the possibilities for evaluation and improvement of oocyte quality by e.g. hormone treatments based on the individual status of the donor (e.g. Ireland *et al*., 2007). Furthermore including a period of FSH withdrawal before aspiration has demonstrated positive effects on IVP results ("coasting"; Blondin *et al*., 2012; Nivet *et al*., 2012). Finally, the possibilities for using plasma anti-mullerian hormone (AMH) as an estimator of donor potential should be further investigated including young females (Silva-Santos *et al*., 2013; Guerreiro *et al*., 2014).

Novel developments in media for *in vitro* production

For many years, home-made media compositions based on commercially available stock solutions have been used for bovine *in vitro* embryo production; Tissue Culture Medium 199 (TCM 199; Sigma-Aldrich), Tyrode's Albumin Lactate Pyruvate (TALP) stocks (Parrish *et al*., 1986) and Synthetic Oviduct Fluid (SOF) with few modifications (Tervit *et al*., 1972; Holm *et al*., 1999) and most of them containing serum. All media for IVF are based on a balanced salt solution, amino acid solutions and pyruvate. Further supplementations are vitamins, EDTA, and metal ion buffers.

With the increasing implementation of IVP of bovine embryos worldwide for commercial use, there is an increased focus on optimizing the yield of blastocysts. Furthermore, increased focus on regulatory restrictions on import/export of embryos cultured in media containing serum due to the risk of spreading pathogens, has increased the wish to supplement the IVP media with bovine serum albumin (BSA) and synthetic serum replacements, instead of serum. Currently a Danish company, EmbryoTrans Biotech ApS, is developing a novel culture medium without any animal originating protein source and strictly synthetic serum based.

As medium for *in vitro* culture (IVC) of the embryos, SOF has been used as a continuous culture medium system. However, an increasing interest at some commercial bovine laboratories to perform sequential culture has evolved. The sequential culture system has been widely used for years in human *in vitro* fertilization (IVF), based on the theory that the embryo has different needs depending on the developmental stage. Hence, the media are composed to provide the optimal support from embryo cleavage stage to the blastocyst stage development (Simon, 2002). However, in human IVF the monoculture medium system is gaining popularity again. The monoculture medium is supplemented with all the required compounds to sustain embryo development to the blastocyst stage, and is based on letting the embryo choose the nutrients and components needed for an optimum development during the entire culture period (Gardner *et al*., 2002). It has been suggested that monoculture medium system is as efficient as the sequential medium system (Macklon *et al*., 2002). Knowing that the embryos worst enemy is the fluctuations, in particularly, of pH and temperature (Swain, 2010), a monoculture medium system has the advantage of decreasing the number of manipulations and the length of time the embryo is out of the incubator. The early embryo produces autocrine/paracrine factors, essential for *in vitro* survival (Gopichandran and Leese, 2006), thus, a monoculture medium system may well be the preferred solution.

With the increasing production of bovine IVP

embryos commercial media are becoming available. The developmental rates and gene expression of IVP blastocysts are affected by the use of different IVP media systems. IVP methods have been evaluated by assessing the health of the offspring born (Wrenzycki *et al*., 2004; Bonilla *et al*., 2014), and recent research has focused on finding a new method, where the quality of the embryo and subsequent calf produced in a certain IVP system, can be evaluated before transfer to the recipient. This research has been centered around finding differences in gene expression and epigenetic modifications between *in vivo* and *in vitro* produced embryos, and a long list of candidate genes, believed to be involved in the critical processes of embryo development, is now available (Wrenzycki *et al*., 2004, 2005; Thompson *et al*., 2007; Wrenzycki *et al*., 2007; Chen *et al*., 2013). Therefore, more studies should be conducted to investigate the correlation to healthy live born offspring from embryo quality in terms of media influence on embryo development such as: gene expression, morphology, kinetics and general blastocyst rates. As abundance of gene expression in itself is not a quality marker the studies should be performed including comparison of *in vivo* produced embryos.

Recently, a preliminary study was published showing increased blastocyst rates, superior embryo quality, and more abundant gene expression in embryos produced in the media system from the Danish company EmbryoTrans Biotech compared to the IVP media system from Minitube Germany (Nielsen *et al*., 2014). The selected genes for their proposed value as quality markers for IVP of bovine embryos that were included in the analysis were: Stress response: HSPA1A (heat shock protein), Glucose transport: SLC2A1 and SLC2A3, DNA methylation: DNMT3A (DNA methyltransferase), Maternal recognition of pregnancy: IFNT2 (Interferon tau), Insulin-like growth factor system (growth): IGF1R, Apoptosis: BAX and BCL1L (pro- and antiapoptotic), G6PD (glucose metabolism) and FASN (fat metabolism).

At oocyte aspiration, by e.g. OPU, the oocytes recovered from the same ovary will be at different stages of prematuration or early atresia.. One approach to deal with this situation has been to induce a temporary arrest of oocyte maturation, where work was done a decade ago (Lonergan *et al*., 2003; Donnay *et al*., 2004; Vigneron *et al*., 2004). Recently, 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. Likewise, questions remain with respect to how the oocytes from very young heifers (older calves) should potentially be treated for optimal results.

Finally, there are many techniques to assess embryonic quality, which include hatching rate analysis, survival to cryopreservation, cell counts of the inner cell mass and trophectoderm, measurement of apoptotic cells and analysis of incidence of chromosomal anomalies (Munoz *et al*., 2014). However, the above procedures have limited practical and wide-spread application for ET in farms. It would be worthwhile to evaluate the pre-implantation embryos for their genetic merit for economic traits and use them in genomic selection – a method that evaluates an animal before it is born. In the accompanying paper by Kadarmideen *et al*. (2015), we describe how genomic screens of pre-implantation embryos could accelerate genetic improvement.

Multi-omics data and application of systems biology to bovine IVP

Animal and veterinary bio-sciences are going through paradigm shift from single low-throughput experiments generating single-layer biological data to often a single integrated experiment, where multi–omics biological data are being generated on individual animals. Modern high-throughput technologies generate data at all levels of the animal biological systems (e.g. genome-wide, transcriptome-wide, metabolome-wide or proteome-wide measurements). This includes reproductive systems and various cell types including ovaries, oocytes, embryos and endometrium. Another angle to -omics data is the emergence of "phenomics", which refers to tens of thousands of phenotypes observed in a single animal instead of a few dozens or a hundred.

Systems biology (SB) approaches, by necessity, involve systematic data collected at all levels of the biological systems and at the individual (animal level) and are aimed at studying interactions between all these levels, but not at one level in isolation, (Kadarmideen, 2008, 2014). It attempts to provide a holistic view of the entire outcome. If reproductive success is an outcome, then it tries to provide an experimental data-driven and hypothesis-based explanation for reproductive success. To achieve this, systems biology collectively models and analyzes these multi-omics datasets using a combination of mathematical, computational biology and bioinformatic principles and tools (Kadarmideen, 2008, 2014). SB is a discipline that iterates between data-driven and hypothesis-driven approaches to understand the whole biological system and provide a complete blueprint of functions of phenotype or a complex disease evolution. Therefore, it requires multi-disciplinary expertise in one team, from mathematical sciences through quantitative genetics to molecular biology.

The term "Systems Genetics", a branch of SB was originally proposed by Kadarmideen *et al*. (2006) which integrate 'omics scale measurements from genome to metabolome to functome through transcriptome and proteome. This systems genetics approach has been applied in livestock (Kadarmideen

and Janss, 2007; Kogelman *et al*., 2014), humans (Civelek and Lusis, 2014) and has been thoroughly reviewed (Li, 2013; Kadarmideen, 2014). One such way is the integration of genomics and transcriptomics by detecting expression QTLs (eQTLs). An eQTL is a genomic region associated with transcript levels, which subsequently affects the phenotype. Systems genetics has been shown to be a powerful method to find important causal and regulatory genes and their variants in predicting biomarkers (for instance reproductive success via a conventional artificial insemination (AI) or embryo transfer (ET).

Several genomic or transcriptomic studies, in isolation, have been conducted to reveal genetic architecture or gene regulatory mechanisms underlying phenotypes or mechanisms that determine pregnancy in bovines, based on transfer of both *in vivo* and *in vitro* produced embryos. There are some large-scale transcriptomic studies in understanding conceptus-maternal communication, which is vital for the establishment and maintenance of pregnancy.

For instance, Bauersach *et al*. (2006) showed that expression of AGRN, LGALS3BP, LGALS9, USP18, PARP12 and BST2 in the endometrium plays a central role in the context of early embryo-maternal communication and pregnancy. Clemente *et al*. (2011) showed differentially expressed genes between day 7 and 13 embryos derived *in vivo* or *in vitro* showing the top 40 up- and down regulated genes on day 13 unique to *in vivo* embryos, unique to *in vitro* embryos, and common to both. (Mamo *et al*., 2011), using RNA Seq technology, generated transcriptomic profiles of bovine conceptuses across the entire pre- and peri-implantation periods (day 7, 10, 13, 16 and 19) and identified clusters of genes associated with blastocyst formation, conceptus elongation, maternal recognition of pregnancy and initiation of implantation. Mamo *et al*. (2011) studied the transcriptome of the uterine endometrium to unravel the genes and pathways governing growth and development of the cattle conceptus. They focused particularly on the time of hatching of the blastocyst from the zona pellucida and its subsequent elongation coincident with the time of maternal recognition of pregnancy.

However, the SB or systems genetics approaches, as described above, for studies of conceptus-maternal communication encompassing multi-omics datasets are lacking. This is exactly one of the focal areas of the Danish-Brazilian GIFT consortium activities (www.gift.ku.dk). As one aspect, the GIFT project envisions to apply transcriptomics and eventually systems genetics approaches to follicular cells to characterize donor cow transcriptomics and systems biology. Briefly, this involves aspiration of oocytes from slaughtered donor cows with production data and subsequent collection and extraction of RNA from mural and cumulus granulosa cells for the RNA seq analysis. Oocytes *in vitro* matured and fertilized with semen from high genetic and low genetic merit bulls are then used to study the effect of genomics-by-sire interaction on blastocyst rates. Furthermore correlation between gene expression in granulosa cells and blastocyst rate, values retrieved for each animal, is analyzed implementing statistical linear models. The analyses will identify differentially expressed genes that can be potential markers for the characterization of donor cows for IVP procedures.

Conclusions and perspectives

The past decade has brought an impressing amount of new knowledge related to bovine OPU-IVP and ET at the biological, physiological, molecular, genetically and especially the practical level. The overall process is basically fairly simple and straightforward, but with the growing knowledge there will be more and more options for making selections along the process. This would be valuable as new traits used to improve overall fertility from a breeding point of view. In addition it also provides an incoming "personalized approach" in bovine assisted reproductive technologies (ARTs) with e.g. individual treatment of the donor based on her status and actual situation; of the bull based on his sperm's reaction to the treatment prior to IVF and of the recipient endometrial status to secure the implantation of the IVP embryo.

Future reflections should take into consideration new traits as decision support tools for reproductive biotechnologies, such as selection of donor cows on embryo production in terms of ability to perform in the IVP laboratory. Quantitative traits such as number of oocytes and number of embryos, and qualitative traits such as quality of oocytes, cleavage rate, development rate, morphology and kinetics of the resultant embryos, are important traits to identify in the fast progressing era of bovine IVP.

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