Anim. Reprod., v.11, n.3, p.183-194, Jul./Sept. 2014

Molecular markers of fertility in cattle oocytes and embryos: progress and challenges

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Abstract

In order for assisted reproduction technologies to improve, better methods to discern eggs and embryos according to their level of developmental competence are urgently required to substitute or complement the subjective morphological selection criteria still broadly in use. Objective and reliable molecular markers of viability have been studied during the last decades as robust options to select the best oocytes and embryos for embryo transfer programs. These molecular methodologies rely mostly on the novel "OMICS" technologies. Among these, transcriptomicsis the primary platform applied so far in animal breeding research mainly due to the possibility to amplify small samples. In addition, biomarkers of competence have not only been instrumental to select the best oocytes and embryos for reproductive technologies, but have also shed light on the intricate molecular physiology leading to the acquisition of developmental capacity within the ovary and on how the embryo manifests this potential during culture. Nevertheless, such molecular profiling usually implies the destruction of the oocyte/embryo hereby preventing the practical use of biomarkers in *in vitro* embryo production and transfer systems. Alternatively, encouraging results have been lately obtained from non-invasive technologies based on biopsies of follicular somatic cells surrounding the developing egg, as well as from metabolic analysis of follicular fluid or spent culture media. This work summarizes the achievements of recent years in the field of biomarkers of competent bovine embryos. The main challenges will be exposed, while the future guidelines will help to comprehend why biomarkers of developmental competence appear promising to take us steps forward in the amelioration of reproductive technologies.

Keywords: bovine, developmental competence, embryo, molecular marker, oocyte.

Introduction

Over the course of the last decades assisted reproductive technologies (ARTs) have been increasingly used to enhance production in domestic animals. Among these methodologies, *in vitro* embryo production (IVP) has allowed the quick propagation of embryos from parents with genetically desired characteristics for the animal breeding industry, and the supply of biological material for reproduction research. Nonetheless, it is clear that oocytes matured *in vitro* can only produce less than half of the blastocysts than *in vivo* systems in cattle unless special care is applied to ovarian preparation prior to oocyte aspiration. Intense investigation of the possible causes of decreased embryo yields following *in vitro* culture (IVC) has highlighted the poor developmental competence, or quality, of the *in vitro*-matured oocytes in comparison to gametes matured *in vivo*, or of those oocytes collected too early or late during the antral phase (Sirard and Blondin, 1996; Merton *et al*., 2003). It is largely accepted that the quality of the female gamete directly depends on specific gene products: transcripts and proteins that are stored in the cytoplasm during oocyte growth and support early development during the transcriptionally inactive period from maturation up to activation of the embryonic genome (Krisher, 2004; Sirard, 2010). Understanding these maternal constituents has not only been used to decipher the intricate regulation of developmental capacity in oocytes and cleavage-stage embryos, but also to obtain markers of fertility during early development. Additionally, embryo culture conditions importantly impact developmental success and the molecular origin of this effect can also be assessed (Lonergan *et al*., 2003b; Cagnone *et al*., 2012; Gad *et al*., 2012). However, the available methods, mostly relying on transcript profiling, involve the sacrifice of the oocyte/embryo in order to perform the molecular analysis (Bols *et al*., 2012; Fair, 2012). This has prompted the search of non-invasive techniques to evaluate competence through the assessment of the somatic compartments of the follicle (Bettegowda *et al*., 2008; Bunel *et al*., 2013; Nivet *et al*., 2013), follicular fluid (Matoba *et al*., 2014), and spent culture media (Sturmey *et al*., 2010; Hemmings *et al*., 2012), while the egg or embryo is preserved for further development. In this review, we will focus on the discovery of fertility markers in bovine through invasive methodologies, on how they have been useful to discern populations of oocytes and embryos according to their developmental capacity, and on the fact that they were crucial to comprehend the complex molecular regulation of quality in gametes and embryos. Remarkably, this has resulted in the deciphering of some of the mechanisms

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Received: April 28, 2014
Accepted: July 3, 2014

responsible for developmental competence acquisition within the ovary throughout folliculogenesis and the way that this potential is then revealed after fertilization, and later on in embryogenesis. Promising non-invasive techniques to analyze developmental competence will also be discussed.

Fertility status of oocytes and embryos

"Developmental competence" or "oocyte quality" is defined as the capacity of the oocyte to successfully mature, be fertilized, and progress through development to form a viable blastocyst that can induce pregnancy and generate a healthy offspring. In cattle it is impossible to transfer all IVP blastocysts to recipient cows. Therefore, the developmental competence of oocytes is usually measured by their capacity to yield blastocysts with adequate morphology and timing for being frozen and potentially transferred (Sirard *et al*., 2006; Mermillod *et al*., 2008). For simplicity "competence" or "developmental potential" will refer here to the ability of either oocytes or early embryos to reach the blastocyst stage. Therefore, the term "markers of competence" will be used in this work as synonym of "markers of fertility".

The requirement for practical standards to identify oocytes and embryos endowed with the highest probability of becoming blastocysts able to induce healthy pregnancies successfully carried to term is one of the central challenges in the clinical application of ARTs. In human, the rate of live births obtained per embryo transferred in utero is only approximately 15% and clinics around the world continue to try to overcome this problem by transferring multiple embryos, which represents one of the major risk issues of ARTs. Nowadays, the arbitrary morphological selection of oocytes to be subjected to IVF or intracytoplasmatic sperm injection, as well as of the embryos to be transferred is still controversial and unable to effectively discern them according to their level of developmental potential. Consequently, human ARTs urgently need far more objective and effective criteria to select, within a group of embryos, the one with the highest developmental capacity in order to transfer a single embryo; or to select the oocyte most likely able to produce such an embryo (Patrizio, 2007; Hemmings *et al*., 2012 for review). A similar scenario occurs in domestic animals, where the blastocyst rate obtained by routine IVP from non-stimulated animals is still limited to 35-45% of the fertilized oocytes (Bettegowda *et al*., 2008; Bols *et al*., 2012; Boni, 2012 for review). It is possible that a male effect exists posterior to syngamy. However, it is widely accepted that the embryo's developmental outcome mostly depends on the intrinsic quality of the oocyte as demonstrated by the fact that existing culture systems can hardly improve blastocyst rates of IVP (Blondin *et al*., 2002; Krisher, 2004; Sirard *et al*., 2006; Sirard, 2010). As in human, the vast majority of cattle oocytes (Blondin and Sirard, 1995) and embryos (Massip *et al*., 1995) selected for ARTs are still graded through subjective morphological standards that do not accurately evaluate the intrinsic developmental capacity of the oocyte/embryo increasing variability of ART procedures between laboratories. In contrast, using objective and more reliable molecular markers of competence derived from the application of "OMICS" technologies could potentially improve ARTs. These biomarkers have been uncovered in recent years through the comparison of oocytes and embryos of extreme levels of competence when applying indirect criteria of developmental potential such as follicular size and stage, morphology, metabolism, maturation media, age of donor, migration speed of oocytes/zygotes under a dielectrophoretic field, cleavage dynamics, or culture stress. Moreover, biomarkers of competence are also useful to elucidate how the mechanisms governing early embryogenesis and acquisition of quality during folliculogenesis are interrelated. Notwithstanding, amelioration in the field of developmental predictive value is still to come (Wrenzycki *et al*., 2007; Boni, 2012; Ruvolo *et al*., 2013 for review) and, together with the current progress in the identification of molecular biomarkers of fertility, applications will be the central subject of this review.

Molecular markers of quality prior to fertilization

Immature oocytes

A robust body of evidence of competence markers is derived from the discrimination of oocytes based on their follicle size, where gametes from larger follicles are of better quality. Several teams have reported that transcripts whose levels varied between GV-oocytes of differing quality pertained to several functional categories including cyclins, histones, and other cell cycle regulators, as well as transcription factors and molecules related to mRNA and protein processing, which are remarkably well represented on the list of potential markers of competence (Robert *et al*., 2000; Donnison and Pfeffer, 2004; Mourot *et al*., 2006; Pfeffer *et al*., 2007; Table 1). In addition, super-stimulation of cows with various protocols of FSH coasting demonstrated that oocyte quality can be modified by systemic factors, which affect ovarian follicular cell physiology first and then reach the developing gamete. Upon applying the appropriate FSH-deprivation period (44/68 h coasting) before ovum pick-up (OPU), it is possible to generate a population of follicles at the right differentiation level and the recovered oocytes resemble those of natural pre-ovulatory follicles possessing optimal developmental capacity. The decrease in circulating FSH is a physiological phenomenon that has been neglected as a tool to control the proper differentiation of follicles (Nivet *et al*., 2012). Notably, the transcriptome signature of GV-oocytes obtained by using this strategy

demonstrated that the main biological functions that varied between oocytes of different quality were related to RNA processing and regulation of chromosome segregation (Labrecque *et al.,* 2013). These authors confirmed 13 mRNA markers of competence (Table 1), including PAIP2, AURKAIP1, CDK1, ENY2, and PMS1 (Labrecque *et al*., 2013). Moreover, further confirmation that hormonal dynamics closely affect the molecular regulation inside the oocyte was demonstrated through the identification of alterations of the transcriptome of immature oocytes collected after FSH-coasting and administration of a GnRH antagonist. Although Labrecque *et al*. (2014) did not report any significant effects of the antagonist cetrotide on blastocyst rates, transcriptomic analysis revealed that cetrotide impaired protein translation capacity, RNA processing, and chromosome segregation in oocytes. Interestingly, such findings coincide with the biological functions identified as being affected in the coasting model, demonstrating how hormonal processes influence the molecular modulation of the gamete's quality (Labrecque *et al*., 2013; Nivet *et al*., 2013).

Assessment of the activity of glucose-metabolizing enzymes has also been successfully applied to distinguish immature oocytes according to their viability. Brilliant cresyl blue (BCB) staining detects the activity of G6PDH, which catalyzes the first step of the pentose phosphate pathway (Gutierrez-Adan *et al*., 2004 for review). BCB+ oocytes, which remain blue due to low G6PDH cytoplasmic activity, are considered as fully-grown gametes of higher quality than BCB- gametes (unstained) that own higher G6PDH activity (Alm *et al.,* 2005; Bhojwani *et al.*, 2007). Bols *et al*. (2012) considered BCB staining as one of the few suitable non-invasive indicators of competence. Notably, classification of immature oocytes by BCB staining followed by transcriptomic analysis has highlighted differential levels of mRNAs (Table 1) related to cell cycle regulation, CCNB1, PTTG1; transcription control, SMARCA5; and protein translation, eIF-, RPL- and RPS-group proteins (Ghanem *et al*., 2007; Torner *et al*., 2008), suggesting the importance of such functions for the potential to develop to the blastocyst stage.

Table 1. Molecular markers of developmental competence in oocytes.

Factor(s)	Type	Stage	Reference(s)
CCNB1	mRNA	GV	Robert *et al*. (2000); Torner *et al*. (2008)
CCNB2, CKS1B, CDC5L, PSMB2, SKIIP, RGS16, PRDX1	mRNA	GV	Mourot *et al*. (2006)
CCNA2, NDFIP1, OCT4, MSX1, ZNF198, SLBP, DNAJA1 (DJA4), GDF9, TRAPPC3	mRNA	GV	Donnison and Pfeffer (2004); Pfeffer *et al*. (2007)
DYNLL1, DYNC1I1	mRNA	GV	Racedo *et al*. (2008)
NASP, SMARCA5, RPS274A, EIF1A, ATP5A1	mRNA	GV	Torner *et al*. (2008)
PTTG1	mRNA	GV	Mourot *et al*. (2006); Ghanem *et al*. (2007)
H2A	mRNA	GV	Caixeta *et al*. (2009)
RPL24, MSX1	mRNA	GV	Ghanem *et al*. (2007)
MATER, YY1, MSY2, PAP, PARN, EIF4E	mRNA	GV	Lingenfelter *et al*. (2007)
HSP70	mRNA	GV	Camargo *et al*. (2007)
CTSB	Protein	GV	Balboula *et al*. (2010)
ATP1A1	mRNA	GV	De Sousa *et al*. (1998)
INHBA, INHBB	mRNA	GV	Patel *et al*. (2007)
ANXA2	mRNA	GV	Costa *et al*. (2006)
PRDX1, PRDX2	mRNA	GV	Romar *et al*. (2011)
G6PDH	Enzymatic activity	GV	Alm *et al*. (2005); Bhojwani *et al*. (2007); Ghanem *et al*. (2007); Torner *et al*. (2008)
BCL2, BAX	mRNA	GV	Opiela *et al*. (2008); Li *et al*. (2009)
RBM42, LSM10, HAUS8, AURKAIP1, CDK1, PAIP2, ENY2, ESCO2, PMS1, ELP4, TFDP1, SFRS7, TAF1A	mRNA	GV	Labrecque *et al*. (2013)
TACC3, SARNP, CTNNBL1	mRNA	GV	Labrecque *et al*. (2014)
CCNB1, GDF9, SOD1, SOD2	mRNA	M-II	Lonergan *et al*. (2003a)
CKS1B, FAM58A, NASP, NUSAP1, CDC91L, SMARCA5, RPL2, RPL8, RPL35, RPLP0, DNMT1, ANXA2	mRNA	M-II	Dessie *et al*. (2007)
AQP3, SEPT7, ABHD4, SIAH2	mRNA	M-II	Katz-Jaffe *et al*. (2009)
PABPNL1	mRNA	M-II	Biase *et al*. (2010)
SFRS14, DDR1, NDUFB6, UQCRH, DUSP6,NDUFS4	mRNA	M-II	Biase *et al*. (2014)
Alanine, arginine, glutamine, leucine, tryptophan	Amino acid	M-II	Hemmings *et al*. (2012)

An attractive approach for quality assessment of the developing female gamete is the analysis of proxies of fertility such as follicular fluid (FF) or biopsied follicular somatic cells (Fig. 1). Molecular characterization of these follicular components represents a non-invasive alternative to investigate the developmental competence of the oocyte without compromising its viability. The most external follicular compartment is thecal cells and Matoba *et al*. (2014) observed that ESR1 and VCAN mRNAs were overexpressed in thecal cells associated with competent oocytes (Table 2). It makes sense that increased levels of VCAN are correlated with higher competence as this proteoglycan may be necessary for ovulation. A larger amount of biomarker data has been derived from granulosa cells. Nivet *et al*. (2013) reported on four putative markers of fertility in these cells and the information was valuable not only because biomarkers of fertility were unveiled, but such findings also shed light on complete molecular pathways (prolactin, growth hormone pathways) related to the events in the granulosa compartment that lead to acquisition of competence of the gamete. In addition, the granulose transcriptomic profiling demonstrated that folliculogenesis in cattle is a highly dynamic and tightly regulated process: The pre-ovulatory differentiation of granulosa cells at the end of follicular growth, which is characterized by angiogenesis, early hypoxia and oxidative stress, contributes to the specific environment required for the oocyte to attain maximum competence (as is the case with 44/68 h FSH coasting). Then, if FSH starvation is extended (92 h), folliculogenesis enters a phase where apoptosis is increased and signs of inflammation appear (Nivet *et al*., 2013), while the quality of the enclosed oocyte suddenly diminishes, exemplifying what could happen if the gamete were not ovulated at the appropriate moment (Labrecque *et al*., 2013). In this sense, the negative influence of such a prolonged coasting period on ovulation rates and oocyte quality has been demonstrated (Dias *et al*., 2013).

Table 2. Molecular markers of developmental competence in follicles.

Factor(s)	Type	Compartment	Reference(s)
VCAN, ESR1	mRNA	Theca	Matoba *et al*. (2014)
IGF2, NRP1, VNN1, KCNJ8	mRNA	Granulosa	Nivet *et al*. (2013)
LHCGR	mRNA	Granulosa	Matoba *et al*. (2014)
HAS, INHBA, EGFR, GREM1, BTC, CD44, TNFAIP6, PTGS2	mRNA	Cumulus	Assidi *et al*. (2008)
CTSB, CTSS, CTSZ	mRNA	Cumulus	Bettegowda *et al*. (2008)
CYP11A1, NSDHL, GATM, MAN1A1, VNN1, NRP1	mRNA	Cumulus	Bunel *et al*. (2013)
TNFAIP6	mRNA	Cumulus	Matoba *et al*. (2014)
L-alanine, glycine, glutamic acid	Amino acid	Follicular fluid	Matoba *et al*. (2014)
Palmitic acid, linoleic acid, total fatty acids	Fatty acid	Follicular fluid	Matoba *et al*. (2014)
Urea	Amino acid metabolite	Follicular fluid	Matoba *et al*. (2014)

Bunel *et al*. (2013) identified six markers of competence in cumulus cells by using the same coasting model as mentioned above (Table 2). Abundance of the CYP11A1 and NSDHL transcripts increased with developmental competence. Since they are involved in progesterone biosynthesis, their highest levels at the moment of optimal quality may indicate that increased levels of this steroid and modulation of its synthesis by CYP11A1 and NSDHL favor acquisition of developmental competence. Similarly, NRP1 and VNN1 mRNA abundance increased in parallel with FSH-coasting and peaked in cumulus cells from over-differentiated follicles, probably reflecting the fact that angiogenesis and the need to deal with oxidative stress are required by the end of folliculogenesis, as NRP1 and VNN1 are involved in such functions, respectively. Interestingly, this expression pattern corresponds to the same profile previously observed by Nivet *et al*. (2013) in granulosa cells, which indicates that the level of angiogenesis and oxidative processes increases in both granulosa and cumulus cells. Additionally, increased GATM expression could reflect the elevated hypoxic condition at the end of folliculogenesis (Bunel *et al*., 2013). These results support the conclusions reached by Assidi *et al*. (2008) that the molecular regulation of cumulus cell function and differenctiation is a complex process that involves events necessary for acquisition of developmental capacity by the oocyte. As a result of an outstanding effort, O'Shea *et al*. (2012) identified markers of competence in cumulus cells and oocytes shared across species and concluded that some of the molecular mechanisms related to competence are conserved. Bettegowda *et al*. (2008) observed that transcript levels of cathepsins varied in cumulus cells according to the viability of the oocyte. In the same report, blastocyst rates were increased by inhibiting cathepsins during IVM. Nevertheless, these authors emphasized the need to understand the molecular basis

of the IVP improvement observed in response to the pharmacological targeting of cathepsins.

Another appealing non-invasive strategy to identify markers of competence is the fingerprinting of the metabolome of FF. Using this method Matoba *et al*. (2014) observed that urea, three amino acids, two fatty acids, and total fatty acid contents varied in the FF associated with oocytes of distinct quality levels (Table 2). Amino acid profiling was particularly predictive of developmental competence. Specifically, T. Fair laboratory's results (Matoba *et al*., 2014) of metabolic analysis of FF were in agreement with previous observations that high levels of urea (De Wit *et al*., 2001), total saturated fatty acids and palmitic acid (Leroy *et al*., 2005) have deleterious effects on oocyte competence during IVM, whereas a surplus of alanine, glycine, and glutamate appear to positively impact development (Sinclair *et al*., 2008). Results of FF metabolome characterization are promising and will contribute to the improvement of maturation media (Matoba *et al*., 2014). However, their practical use in commercial IVP programs is currently challenging because more information is still needed to establish a clear correlation of FF metabolites and oocyte fertility status (Revelli *et al*., 2009; Bols *et al*., 2012).

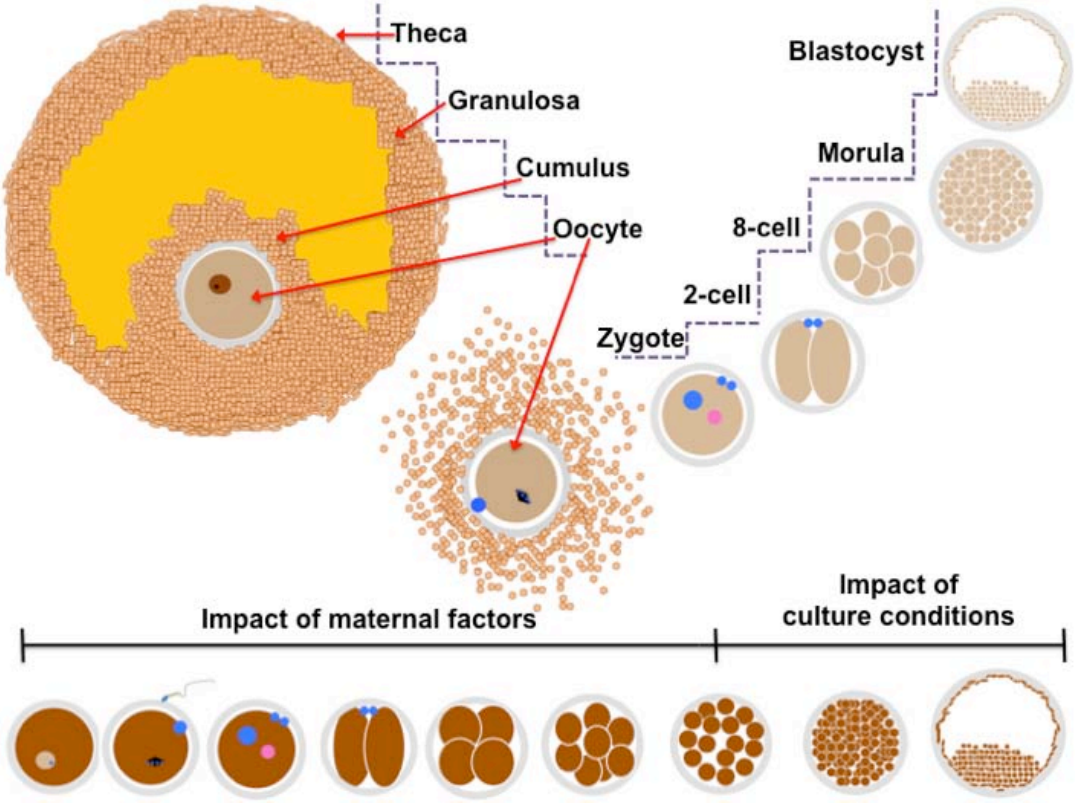

Figure 1. Multi-step molecular markers of competence.

Mature oocytes

Using transcriptomic analysis, Biase *et al*. (2014) identified twenty-nine putative mRNA markers of quality in bovine mature oocytes (Table 1). It is noteworthy that the most variable biological functions between oocytes of high and low developmental competence were RNA processing and translation as observed in GV-oocytes (Labrecque *et al*., 2013). The metabolomic analysis, of spent IVM media identified variations in the capacity to turnover alanine, arginine, glutamine, leucine, and tryptophan between oocytes of distinct developmental competence (Hemmings *et al*., 2012). Overall M-II oocytes of decreased quality had higher amino acid turnover rates. This is in agreement with the quiet embryo hypothesis (Leese, 2002; Baumann *et al*., 2007; Leese *et al*., 2008), which states that less competent embryos have major levels of metabolic activity. Hemmings *et al*. (2012) extended the notion of metabolic quietness as a sign of higher developmental potential to oocytes and suggested that the metabolism of an oocyte or cleavage stage embryo could be a reflection of its stored maternal transcripts.

Molecular markers of quality in embryos

From fertilization to embryonic genome activation

In this section, biomarkers of competence found in zygotes, as well as in 2-cell and 8-cell embryos will be summarized. Prior to embryonic genome activation (EGA), in the absence of *de novo*

transcription, the embryo still depends on maternal stocks of mRNAs and proteins and on the metabolic machinery inherited from the oocyte for its development (Fig. 1; Krisher, 2004; Marlow, 2010; Sirard, 2010). Considering that it is generally accepted that EGA occurs in cattle at the 8-16 cell stage (Barnes and First, 1991; Memili and First, 1998, 1999), the first cleavages provide a relatively long time-span where biomarkers of competence are likely exclusively from maternal origin (Lechniak *et al*., 2008; Orozco-Lucero *et al*., 2014). Analysis of zygotes of distinct levels of fertility has unveiled seven potential biomarkers of competence (Table 3), most of them related to the functions of cell cycle regulation: NASP, AURKA, and IQGAP1; and transcription regulation: DDX10, DNMT1, and SMARCA5 (Dessie *et al*., 2007). Amino acid (turnover of overall amino acids) profiling of spent culture medium confirmed that the most metabolically inactive zygotes were the most likely to reach the blastocyst stage (Sturmey *et al*., 2010). These findings are in agreement with those of Hemmings *et al*. (2012) described above concerning metabolically quiet oocytes.

One of the new parameters that arise upon fertilization that can be used to evaluate developmental competence is embryonic cleavage dynamics. It is generally accepted that early-cleaving embryos produce higher blastocyst rates than their slow-cleaving counterparts (Lechniak *et al*., 2008; Orozco-Lucero *et al*., 2014). Although the exact nature of this phenomenon and the way in which it impacts developmental capacity, or reflects it, is still not fully understood, the most plausible hypothesis is that the elevated competence accompanying fast embryonic division is mostly due to intrinsic characteristics of the oocyte from which the cleaving embryo originates. The fact that embryonic cleavage speed is correlated with developmental capacity has been observed across species and timing to the first zygotic division has been used as a parameter to separate embryos of variable fertility status and to try to identify the molecular mechanisms underlying early cleavage (Lechniak *et al*., 2008). Initial efforts to unveil markers of competence in 2-cell cattle embryos of differing cleavage speed have identified transcripts of differential abundance levels between fast- and slow-cleaving embryos. These transcripts (Table 3) are related to various biological functions such as structure: CX32, CX43, PKP1 (Brevini *et al*., 2002; Gutierrez-Adan *et al*., 2004); glucose metabolism: IDH, G6PDH, GPI, HK1, (Lequarre *et al*., 1997; Dode *et al*., 2006); transport: GLUT1 (Lequarre *et al*., 1997); signaling: BMP15, PED, IGF2, IGF1R, IFNT, FS, INHA, INHBB (Fair *et al*., 2004a, b; Gutierrez-Adan *et al*., 2004, Patel *et al*., 2007); oxidative stress: SOD2 (Gutierrez-Adan *et al*., 2004); cell cycle regulation: CCNB1, (Fair *et al*., 2004b; Bermejo-Alvarez *et al*., 2010); transcription control: OCT4, YEAF1 (Brevini *et al*., 2002; Dode *et al*., 2006); DNA packaging: H2A, H3A (Fair *et al*., 2004b; Dode *et al*., 2006, Mourot *et al*., 2006); protein regulation: CTSB, TCP1 (Dode *et al*., 2006); transcript processing: PAP, PARN (Brevini *et al*., 2002); and DNA repair: RAD50 (Dode *et al*., 2006). In an ingenious study, Held *et al*. (2012) analyzed the transcripts from one of the blastomeres of 2-cell embryos resulting in blastocysts at either high or low rates upon individual culture of the remaining sister blastomere. The transcriptomic contrast uncovered that NRF2-mediated oxidative stress response and oxidative phosphorylation were the main biological functions varying between competent and unviable blastomeres. Ten candidate markers of fertility were validated by RT-qPCR through an independent model of time to the first zygotic cleavage (Held *et al*., 2012). Our laboratory compared fast- and slow-dividing 2-cell embryos by transcriptomic analysis and identified cell cycle regulation, DNA damage response, RNA processing, transcription control, and protein degradation as the main biological functions differing between 2-cell embryos of variable developmental fitness (Orozco-Lucero *et al*., 2014). Ten of the candidate markers of competence that were confirmed by RT-qPCR were involved in crucial functions such as DNA damage response: ATM, ATR, MRE11A, MSH6, CTNNB1; cell cycle: APC, PCNA, CENPE; and transcription control: TAF2 (Orozco-Lucero *et al*., 2014). The finding that the most viable 2-cell embryos had higher levels of mRNAs related to DNA damage response could either mean that such embryos have suffered less DNA offenses and therefore had not translated these mRNAs, or that competent embryos are better equipped to deal with DNA damage prior to EGA. It is tempting to speculate that there is a possible association between reduced DNA damage in 2-cell embryos and quiet metabolism. In fact, Sturmey *et al*. (2009) correlated increased levels of DNA damage in pig, cow, and human embryos with elevated metabolic activity manifested as high amino acid turnover. These authors speculated that this could be due to the fact that the less viable embryos with more DNA damage attempt to avoid developmental arrest by repairing it. Consequently, the least competent embryos need to increase their metabolism (including processes involving amino acid turnover) to perform this additional molecular 'work' compared to healthier and metabolically quieter embryos. Moreover, our results with 2-cell embryos were consistent with the report of Labrecque *et al*. (2013) where the most viable GV-oocytes seemed to be better prepared to regulate meiosis and process mRNA. Thus, an improved maternal stock of transcripts related to cell cycle regulation in oocytes and cleavage-stage embryos might reduce the risk of aneuploidy, which is a major cause of embryonic arrest (Pers-Kamczyc *et al*., 2012). One of our major hypotheses concerning oocyte quality is that the most competent oocytes and their derived embryos are better supplied with maternal molecules that will help them go smoothly through EGA, when modulation of mRNA

processing, transcription, cell cycle, and protein translation/degradation are key events (Sirard, 2010). This notion makes sense in the light of the multiple biomarkers of competence related to these functions found in oocyte/embryo compartments even prior maturation. Ripamonte *et al*. (2012) reported differential abundance of PI3KCA and ITM2B mRNAs between early- and late-cleaving 8-cell embryos (which are approaching the EGA time point). Both molecules are related to apoptotic mechanisms and their presence might reflect the need for programmed cell death regulation in bovine embryos at the 8-cell stage and beyond.

Table 3. Molecular markers of developmental competence in embryos.

Factor(s)	Type	Stage	Reference(s)
NASP, AURKA, IQGAP, SMARCA5, DDX10, DNMT1, RGS2	mRNA	Zygote	Dessie *et al*. (2007)
Total amino acids	Amino acid	Zygote	Sturmey *et al*. (2010)
CCNB1	mRNA	2-cell	Bermejo-Alvarez *et al*. (2010); Fair *et al*. (2004b)
TCP1, RAD50, YEAF1 (RYBP), CTSB, IDH	mRNA	2-cell	Dode *et al*. (2006)
H2A	mRNA	2-cell	Dode *et al*. (2006); Mourot *et al*. (2006)
H3A, BMP15	mRNA	2-cell	Fair *et al*. (2004b)
OCT4, PAP, PARN, HSP70, PKP1, CX43, CX32, PLAT	mRNA	2-cell	Brevini *et al*. (2002)
GLUT1	mRNA	2-cell	Lequarre *et al*. (1997); Brevini *et al*. (2002); Oropeza *et al*. (2004)
PED	mRNA	2-cell	Fair *et al*. (2004a)
CX43, IGF2, IGF1R, IFNT, GLUT5, SOD2	mRNA	2-cell	Gutierrez-Adan *et al*. (2004)
FS, INHA, INHBB	mRNA	2-cell	Patel *et al*. (2007)
G6PDH	mRNA	2-cell	Lequarre *et al*. (1997); Gutierrez-Adan *et al*. (2004)
GPI, HK1	mRNA	2-cell	Lequarre *et al*. (1997)
ATF1, BSG, CAT, MAPK14, NDUFS1, PRDX1, PRDX6, SFRS12, SYCP3, TEAD1	mRNA	2-cell	Held *et al*. (2012)
ATM, ATR, CTNNB1, MSH6, MRE11A, PCNA, APC, CENPE, GRB2, TAF2	mRNA	2-cell	Orozco-Lucero *et al*. (2014)
PI3KCA, ITM2B	mRNA	8-cell	Ripamonte *et al*. (2012)
CTNNB1	Protein	Morula	Modina *et al*. (2007)
PTTG1, MSX1, TNF, EEF1A1, PGK1, AKR1B1, CD9, KRT8, OCLN, COX2, CDX2, ALOX15, BMP15, PLAU, PLAC8	mRNA	Blastocyst	El-Sayed *et al*. (2006)
CX43	mRNA	Blastocyst	Nemcova *et al*. (2006)
Aspartic acid, glutamic acid asparagine, histidine, threonine, arginine, alanine, tyrosine, methionine, valine, phenylalanine, isoleucine, leucine, lysine	Amino acid	Blastocyst	Sturmey *et al*. (2010)
PLAC8, HMGCS1, LDHB, RPS4X, PLAU, NTR	mRNA	Blastocyst	Côté *et al*. (2011)
PNRC2, CLGN, MDH2, HSPE1, COX7B, ALDH7A1, POMP, ATPIF1, HSPA14, COX5A, CDC2	mRNA	Blastocyst	Gad *et al*. (2011)
FL405, HSPD1, S100A10, PLAC8, BMP15, KRT8, RGS2	mRNA	Blastocyst	Ghanem *et al*. (2011)
IGFBP7, HIF1A, TKTL1, PPARG, LDHA, TNFRSF1A, TP53BP2, VIM, JAM2, ADAMTS1	mRNA	Blastocyst	Cagnone *et al*. (2012)
MT1A, DNMT3A, IGFBP7	mRNA	Blastocyst	Plourde *et al*. (2012)
MSMO1, ABCC2, OCT4, PGRMC1, NFE2L2, CYP51A, SFN, HMOX1, PTGS2, PRDX1, HSD17B11, SOD1, IFNT, RARRES1, ANXA1	mRNA	Blastocyst	Gad *et al*. (2012)
ARRB2, SERPINE1, IGFBP7, TPI1, TKDP1, IFNT, GCSH	mRNA	Blastocyst	Cagnone and Sirard (2013)
APEX, CLDN6, LDLR, HMGCS1	mRNA	Blastocyst	Cagnone and Sirard (2014)
HSD3B1, SREBF2, SLC23A1, MYL7, MAPK8, FADS1, ACTA2, DNAJC15	mRNA	Blastocyst	Gad *et al*. (2014; personal communication)

Morulae and blastocysts

Very few biomarkers of fertility have been identified in bovine embryos at the morula stage. The dynamic distribution of the CTNNB1 protein in morulae is associated with fast embryonic cleavage and high competence, as pointed out by Modina *et al*. (2007). In contrast, several markers of fertility have been identified so far at the blastocyst stage. Unfortunately the opportunities to transfer IVP-blastocysts to assess their final capacity to establish pregnancy are rare. Therefore, molecular markers at the blastocyst stage have been used mostly to characterize how blastocysts modify their quality and how they react to different *in vivo* or *in vitro* conditions, or to specific stress conditions achieved by culture medium supplementation (Fig. 1). By using non-invasive amino acid profiling of *in vivo*-generated and IVP-blastocysts, it was corroborated previous findings at the M-II and zygote stage, where the most metabolically quiet oocytes/embryos had higher competence. This time, it was observed that the IVP- blastocysts, likely less competent, consumed more amino acids than their *in vivo*-generated counterparts (Sturmey *et al.*, 2010). In relation to medium supplementation, Cagnone *et al*. (2012) tested hyperglycemic culture conditions and observed that the resulting blastocysts were affected in their extracellular matrix signaling, calcium signaling, as well as energetic metabolism, while such modified gene expression was also related to the Warburg effect (induction of aerobic glycolysis) as if these blastocysts were activating pathways related to cancer and diabetes. The effects of oxidative stress have been examined in culture too by supplementation with two pro-oxidant agents, AAPH and buthionine sulfoximine, which differentially impacted on blastocysts biological functions such as oxidative stress, energy metabolism, glycine metabolism, cellular homeostasis, and inflammatory response. Importantly, this work allowed us to observe that the most metabolically inactive embryos seemed to better survive to oxidative stress (Cagnone and Sirard, 2013). Subsequently, Cagnone and Sirard (2014) unveiled the changes triggered by supplementation of the culture medium with different proteins and lipids. The expression of genes related to ceramide-induced oxidative stress, inflammation, and cholesterol metabolism was altered in response to distinct supplementation and the expression of a pair of pluripotency-associated genes (APEX, CLDN6) was also modified (Table 3). A different perspective on how culture conditions affect early development arose from the comparison by transcriptomic analysis of blastocysts developed in the reproductive tract of super-stimulated cows with those cultured in the tracts of non-stimulated recipient cows (originally transferred to the oviduct as 2-4 cell embryos). Eleven candidate markers were validated in this study, and day 7-blastocysts flushed from the uterus of super-ovulated animals had higher expression of genes involved in transcription, translation, stress response, oxidative stress, oxidative phosphorylation, as well as cellular and metabolic activity (Gad *et al*., 2011). Furthermore, Gad *et al.* (2012) unraveled the effects of the surrounding environment on embryo development by comparing blastocysts obtained from alternation of *in vivo* and IVC (switching at either EGA or morula stage) against embryos completely cultured *in vitro* or *in vivo*. Whereas the oocyte maturation environment (*in vivo/in vitro*) importantly impacted developmental competence, changing culture conditions up until around the time of EGA did not affect blastocyst rates. However, changing culture conditions had a marked impact on transcript profiles demonstrating the sensitivity of embryos to their environment around the time of EGA. In this survey, oxidative stress (including NRF2-mediated oxidative stress response) and lipid metabolism were the most altered biological functions. Outstandingly, negative environmental effects occurring as early as by the time of EGA could influence pluripotency of the analyzed blastocysts, as observed by the variable expression of OCT4 (Gad *et al.*, 2012). In Gad *et al*. (2014, Faculty of Agriculture, Cairo University, Institute of Animal Science, University of Bonn, personal communication), the culture environment alternation occurred around the morula stage and transcriptional analysis revealed that cell death, lipid metabolism, NRF2-related oxidative stress, integrin signaling, and TNFR1/2 pathways were the most affected biological functions between each of the three groups of stressed embryos and the golden standard group fully cultured *in vivo*. In this study, eight putative markers of developmental competence were confirmed by RT-qPCR (Table 3). Noticeably, the authors suggested that embryos that develop to the blastocyst stage under harsh *in vitro* conditions try to adapt to the challenging culture environment and as a consequence their transcriptome is modified. Interestingly, a potential carry-over effect of the detrimental culture environment can affect the pluripotency status of the resulting embryos given that the mRNA level of the transcription factor KLF4 was affected. In summary, the previous works have helped to better understand how embryos adapt to different culture conditions. Shortly after EGA a variable culture environment not only prompts remarkable metabolic changes in embryos (Gad *et al.*, 2011; Cagnone *et al.*, 2012; Cagnone and Sirard, 2013), but also modifies the expression of pluripotency-related genes (Gad *et al.*, 2012, 2014, personal communication; Cagnone and Sirard, 2014).

In spite of the fact that is difficult to find studies in cattle that correlated molecular biomarkers with the ultimate measure of developmental competence (calf delivery), two important surveys must be mentioned. In the first study, biopsies of IVP-blastocysts were transcriptome-profiled, while the rest of the embryo was transferred. Blastocysts that

produced a calf were enriched in transcripts related to implantation and signaling. In contrast, embryos unable to generate pregnancies had increased levels of mRNAs (Table 3) associated with inflammation, protein binding, transcription, cell cycle control, and implantation inhibition (El-Sayed *et al*., 2006). Subsequently, with a similar strategy but this time using *in vivo*-derived blastocysts, Ghanem *et al*. (2011) reported that embryos that produced a calf were enriched in BMP15, KRT8, RGS2, as well as in the marker of placental development and embryo-maternal interaction PLAC8; whereas blastocysts unable to establish a gestation had higher FL405 and HSPD1, which are associated with mitochondrial function and stress, respectively. Interestingly, in this report, the list of markers that differed between blastocysts able and unable to produce a pregnancy was compared with the list from El-Sayed *et al*. (2006) in order to find shared genes. Although three markers had no correspondence, probably due to the influence of the different culture environments (*in vivo/in vitro*), eighteen markers were in agreement, implying that blastocysts capable to bring pregnancy to term have similar gene expression patterns in spite of the culture environment. Therefore, both studies demonstrated the feasibility of using gene markers of implantation in cattle.

Conclusion

The ability to discern populations of oocytes/embryos of different levels of developmental capacity has been a holy grail pursued by reproductive scientists for decades. The work to achieve this now appears to be going in the right direction with the use of powerful "OMICS" technologies. Distinguishing gametes and embryos according to their fertility status is not the only major benefit of fertility markers. These molecules are helping to unravel the intricate modulation of the acquisition of competence during early development, whether in any of the follicular compartments (and their interactions) or in the developing embryo. Such molecular markers are also instrumental at comprehending the way in which the surrounding environment impacts early development and what are the possible resilience mechanisms of embryos in relation to their milieu.

The main challenges for a practical application of biomarkers of fertility in oocytes and embryos are: 1) to generate a standard and consensual list of competence markers and avoid the confusion arising from the large amount of data and the long list of candidate markers; 2) to improve the identification of non-invasive biomarkers; 3) to integrate all the information from different sources and developmental stages into a broad and comprehensive scheme of the molecular physiology leading to developmental potential acquisition; 4) to utilize this knowledge to ameliorate the protocols of super-stimulation/oocyte recovery and IVC in order to provide through systemic (e.g. hormonal super-stimulation) or local targeting (e.g. IVC media supplementation) the developing oocyte with conditions more reflective of the natural microenvironment.

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