Comprehensive cross production system assessment of the impact of *in vitro* microenvironment on the expression of messengers and long non-coding RNAs in the bovine blastocyst

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Abstract

In vitro production (IVP) of cattle embryos over the past two decades has revealed several negative impacts that have been attributed to the artificial microenvironment. Studies on embryos produced *in vitro* clearly point to aberrant gene expression levels. So far, the causal association between phenotype and measured gene expression has not led to substantial improvement of IVP systems. The aim of this study was to generate a unique dataset composed of microarray-derived relative transcript abundance values for blastocysts produced in ten *in vitro* systems differing primarily in culture medium formulation. Between-group comparisons determine the level of overall similarity among systems relative to *in vivo* reference embryos. The use of the dataset to contrast all *in vitro* treatments with the *in vivo* blastocysts pointed to a single common gene network. The 'boutique' array contained a panel of novel uncharacterized transcripts that were variably expressed depending on the medium in which the blastocysts were produced. These novel transcripts were differentially expressed in blastocysts even as carryover from conditions encountered 7 days earlier during oocyte maturation. All of the selected novel candidates thus expressed were from intergenic regions. The function of this long non-coding RNA remains unknown but clearly points to an additional level of complexity in early embryo development.

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Introduction

The extended use of *in vitro* production (IVP) techniques for cattle embryos over the past two decades has proven their ability to produce seemingly healthy offspring. In cattle, IVP is primarily employed to palliate infertility and increase the birth rate of high genetic merit animals (Bousquet *et al.* 1998, 1999, Durocher *et al.* 2006). The sustainability of early development *in vitro* widely differs across species. Various systems tested and developed for cattle are now considered efficient for routine commercial production of embryos. The standard reference in artificial reproductive technologies applied to dairy animals is multiple ovulation embryo transfer in which a super-ovulation regimen leads to the production of multiple embryos recovered by uterine flushing a week after artificial insemination. In comparison, recovery of immature oocytes after ovarian stimulation (ovum pickup) that are submitted to IVP can generate four times the amount of transferable embryos by allowing the donor cow to recover among each of several rounds

of ovum pickup while embryos are produced *ex vivo* (Bousquet *et al.* 1998, 1999, Durocher *et al.* 2006).

However, the artificial fertilization and early embryo development environment has several shortcomings, that have recently been identified. Among the best-described negative impacts of IVP are the birth of abnormally large calves or 'large offspring syndrome' (Young *et al.* 1998, McEvoy *et al.* 2000), the skew in the sex ratio in favor of males (Kimura *et al.* 2005, 2008), and increased sensitivity to cryopreservation (Rizos *et al.* 2003, 2008), which hinders the export potential of embryos obtained *in vitro*. Due to these problems, a large number of studies are conducted to characterize blastocysts produced *in vitro* and comparing them to their *in vivo* counterparts to identify the causes of the deleterious effects of IVP and to improve *in vitro* conditions for embryo development (Wrenzycki *et al.* 1998, 2001, Niemann & Wrenzycki 2000, Knijn *et al.* 2002, 2005, Lonergan *et al.* 2003, Rizos *et al.* 2003, 2008, Gutierrez-Adan *et al.* 2004, Mohan *et al.* 2004, McHughes *et al.* 2009, Purpera *et al.* 2009). As is often

Figure 1 *In vitro* culture conditions: the oocyte fertilization procedure was identical for all *in vitro* conditions. TCM, tissue culture medium 199; SOF, synthetic oviductal fluid. Treatment 7 served as a control under suboptimal conditions (i.e. without protein supplementation). Co-culture conditions involved the support of BRL cells grown in the presence of serum. *In vivo* embryos were collected by uterine flushing 7 days after insemination. The blastocyst yield is a subjective evaluation ranking the systems on their potential to produce high numbers of healthy looking blastocysts.

the case for samples of very small mass such as embryos, most of these studies have focused on gene expression. Significant differences in transcript abundance between *in vitro* and *in vivo* embryos have repeatedly been observed using both the candidate gene approach and the comprehensive methods such as microarrays.

A certain degree of plasticity is expected in embryogenesis even *in vivo*, as the developing embryo adapts to normal changes in the uterine environment, which are not necessarily identical from one mother to the next or even from one gestation to the next. It is, therefore, also expected that *in vitro* embryos deviate to some extent from the gene expression patterns observed in their *in vivo* counterparts. Based on the published gene expression studies, it should be possible to define the boundaries of this plasticity and identify key gene pathways involved in embryo development. However, large methodological differences among studies prevent the comparison of their gene lists and their integration into a comprehensive model of embryonic development (Gilbert *et al.* 2010, Robert 2010, Seli *et al.* 2010). Linking the observed variations in transcript abundance with the observed phenotypes has been extremely challenging. A database generated from studies based on

a standardized methodological approach would facilitate the required comparisons. Therefore, the objective of this study was to generate transcript abundance data from blastocysts produced under a wide range of *in vitro* conditions. Using a 'boutique' microarray, we were able to contrast ten IVP systems, distinguished by culture media composition and protein supplementation during oocyte maturation or embryo culture, with each other and with *in vivo* reference embryos. The comparisons between treatment groups offer possibilities for better definition of the concept of embryonic plasticity.

Results

Performances of the different embryo production systems

The ten embryo production systems provided considerable variations in embryo yield (Fig. 1). Blastocyst yield calculated as the percentage of matured oocytes that developed into blastocysts after fertilization is presented in Table 1. The yield ranged from 30 to 40% for the best-performing systems to 10–20% for the least. The overall appearance of typical blastocyst cohorts found on day 7.5 of culture for most of the IVP systems is presented in Fig. 2.

Experimental treatments 2–5 are synthetic oviductal fluid (SOF)-based systems in which BSA and serum supplementation were alternated for the *in vitro* maturation and *in vitro* culture steps to produce all plausible combinations (Fig. 1). The blastocyst yields were similar (Table 1) and all treatments produced large, fully expanded blastocysts (Fig. 2). However, in some treatments (most obvious in treatment 7), the morphology of the average blastocyst exhibited some differences being smaller and darker (Fig. 2). In experimental treatment 6, serum was added on the last 2 days of embryo culture and thus after embryonic genome activation in an attempt to determine whether fully active embryonic cells could better use this hormone-rich supplement. This treatment did not prove beneficial and resulted in a poor blastocyst yield ($\approx 5\%$ blastocyst yield on day 7.5 of culture). Due to its poor performance, this treatment was dropped from

Table 1 Embryo production yields for different *in vitro* systems.

Nos ^a	IVP treatment		COCs	Cleaved (%)	Eight-cell ^b (%)	Blastocyst ^c (%)	Hatched ^c (%)
	IVM	IVC					
2	SOF-BSA	SOF-BSA	65	55 (84.6)	41 (63.1)	23 (35.4)	11 (16.9)
3	SOF-serum	SOF-BSA	65	57 (87.7)	39 (60.0)	19 (29.2)	8 (12.3)
4	SOF-BSA	SOF-serum	65	60 (92.3)	47 (72.3)	25 (38.5)	6 (9.2)
5	SOF-serum	SOF-serum	65	58 (89.2)	39 (60.0)	24 (36.9)	2 (3.0)
7	SOF	SOF	56	48 (85.7)	39 (69.6)	18 (32.1)	6 (10.7)
10	TCM-serum	SOF-BSA	154	137 (90.0)	95 (61.7)	53 (34.4)	15 (9.7)
11	TCM-serum	TCM-serum	187	148 (79.1)	90 (48.1)	38 (20.3)	0 (0)

^aData for treatments 6, 8, and 9 are incomplete and were not compiled. ^bEight-cell determined 3 days after fertilization. ^cBlastocyst and hatching rates determined 7.5 days after fertilization.

Figure 2 Blastocyst morphology. The different IVP systems resulted in blastocysts of different sizes. Variance in cohort homogeneity can also be observed. Phase contrast images at 400 $\times$. Scale increment = 100 μ m. Media are the same as in Fig. 1.

microarray analyses once embryo pools were collected. The control treatment (7), which did not include any protein supplementation and was intended as suboptimal, gave a blastocyst yield (32.1%) comparable to the moderate to best-yielding systems (Table 1). However, these embryos appeared to be of deviant phenotype, the blastocysts being on average, smaller, and underdeveloped in comparison with those from other high-yielding systems (Fig. 2).

The use of tissue culture medium 199 (TCM) during oocyte maturation led to acceptable yields of embryos (treatments 9 and 10), although its use to support embryo development was detrimental (treatment 11) unless embryos were co-cultured with somatic cells (treatment 9). Performance data collection was incomplete for

treatments 8 and 9. They, however, performed as expected for co-culture supported systems, e.g. $\approx 30\%$ of blastocyst yield on day 7.5 of development. These Buffalo rat liver (BRL) cells have widely been used in commercial settings as the supporting monolayer of somatic cells due to their appropriate sanitary status. The presence of somatic cells, even when non-oviductal in nature, is known to condition medium by neutralizing detrimental factor in serum. The different supplementations also suggest that serum is unnecessary for embryonic development in SOF medium (treatments 2, 3, and 7) and even detrimental (treatment 6).

Microarray data validation

Although the blastocyst yield is commonly used as an indicator of IVP success, as it is a non-invasive determination of the overall efficiency of the procedure, this parameter provides very little information on the characteristics of the produced embryos. We, therefore, turned to our boutique microarray to determine the expression profile of the embryos produced in the ten IVP systems described above in addition to that of *in vivo* embryos.

To test the robustness of the microarray dataset, validation was undertaken by qRT-PCR using a panel of candidates. The aim of the validation was to determine whether the relationship between treatments based on RNA abundance values observed by microarray could be confirmed. Comparisons of qRT-PCR transcript abundance and microarray values indicate similar patterns for a high proportion of the candidates for the three tested treatments (Fig. 3). An expressed sequenced tag corresponding to a novel transcribed region (NTR) matching a still unassigned contig on the bovine genome was also tested (Fig. 3). The similar profiles suggest that the dataset does in fact reflect the relationship between IVP systems. As such, analyses were performed to compare the IVP systems overall in order to highlight relationships between them in addition to their distance from the *in vivo* embryos.

Comparative assessment of blastocyst gene expression across IVP systems

Hierarchical clustering of microarray data indicates that the closest treatment to the *in vivo* reference is TCM/TCM co-culture (treatment 9), which also produced one of the best blastocyst yields (Figs 1 and 4). In contrast, when *in vitro* maturation (IVM) was supported with serum and the culture period supplemented with BSA (treatments 3 and 10), embryos produced expression profiles that clustered away from the main grouping, suggesting a potentially negative impact of the transition from a serum-free to a serum-supplemented environment. The suboptimal control treatment SOF/SOF (7) grouped with another system leading to a poor

Figure 3 Validation of microarray dataset by qRT-PCR: contrasts were made by comparing the *in vitro* systems with blastocysts of *in vivo* origin as the reference. Additional pools of embryos were produced for the qRT-PCR measurements. BSA-BSA = SOF-BSA/SOF-BSA (treatment 2); serum-BSA = SOF-serum/SOF-BSA (treatment 3).

yield (11) and unexpectedly with the SOF-BSA/SOF-serum treatment (4), which showed high variability among biological replicates as indicated by the size of the cluster (Fig. 4). This variability detected through gene expression analysis could be indicative of heterogeneity in the embryos produced using this system.

Although treatment 6 (SOF-BSA/SOF-BSA 5d + SOF-serum 2d) resulted in a low blastocyst yield, the RNA abundance profile was close to the *in vivo* reference (Fig. 4A) and grouped with several other SOF-based treatments (2, 5, and 8) (Fig. 4B). The SOF-serum/SOF-serum (treatment 5) produced the greatest variability between biological replicates, observed as the large diameter of the cluster representation (Fig. 4B). Taken together, these profiles indicate that the addition of serum to SOF medium during embryo (treatments 4 and 5) culture leads to more heterogeneous groups of blastocysts compared with BSA-supported or co-culture-supported *in vitro* culture (IVC).

Impact of culture condition on gene expression of candidates

At the single gene level, clear differences between treatments and groups of treatments can be seen in the

clustering of the differentially expressed genes that harbor the most significant *P* values (Fig. 5). The most striking result is the presence of numerous 'no match' candidates, which represent NTRs that were positioned on the genome but not found associated with any known genes. This straightforward analysis suggests that novel transcripts represent 18/100 of the candidates most significantly influenced by *in vitro* conditions in this study.

Impact of embryo culture conditions on the expression of mitochondrial and uncharacterized transcripts

These observations were confirmed by measuring the abundance of several NTR across a set of SOF-based IVP systems. The results show that this novel RNA class is greatly influenced by the microenvironment, with some treatments rendering the targets undetectable (Fig. 6). The measurements exhibited considerably more variance between replicates than for the protein-coding candidates. The features on the microarray also allowed detection of transcripts originating from both the nuclear and the mitochondrial genomes. We found that IVP conditions also impact mitochondrial gene expression (Fig. 6), clearly indicating that culture conditions influence every level of gene expression including

Figure 4 Between-group analyses: (A) cluster dendrogram of the relationship between embryo production systems (reference to treatment number presented in Fig. 1 is indicated). (B) Relationship distances are based on eigen values between production systems. The size of the cluster is indicative of the degree of variation between biological replicates. The media are as in Fig. 1. The *in vivo* embryos were used as the reference.

protein-coding, non-coding, and transcribed elements found in both the nuclear and the mitochondrial genomes.

Functional analysis of gene expression discrepancies contrasting different IVP systems

The microarray dataset generated in this study offers the flexibility to contrast different treatments or groups of treatments. The aim of one of the contrasts of interest was to evaluate the impact of serum (added either during IVM or to the embryo culture medium as a supplement) by identifying transcript abundance fluctuations.

As illustrated in Fig. 7, the main pathway identified suggests that embryos produced from oocytes matured in the presence of serum and later supported by BSA during culture display differential expression of genes involved in protein synthesis pathways, while the presence of serum during embryo development has an impact on the poly-ADP ribose polymerase pathway. The top three biological functions associated with the gene network presented in Fig. 7 are cell death, gene expression, and lipid metabolism. Interestingly, the lower hatching rate observed in treatments 4 (SOF-BSA/SOF-serum) and 5 (SOF-serum/SOF-serum) compared with the serum-free system (2) may be attributable to increased cell death (Table 1). These functions also fit with the most recognized impact of serum supplementation, which is lipid-over accumulation. A list of all significant biological functions influenced by the presence of serum during either IVM or IVC is presented in Fig. 8. Many of these functions are relevant to the cellular interactions and organization that form the basis of blastocyst formation.

Notwithstanding the diverse nature of the IVP systems in this study, data from all treatments were pooled and contrasted with the *in vivo* reference in order to find differentially expressed transcripts across all treatments and thus common to any *in vitro* conditions. This analysis generated a short list of 33 candidates including nine novel transcripts and six ESTs yet to be sequenced. We note with interest that 15 of the remaining candidates bearing an official gene annotation and function-related information pointed to a single cellular pathway (Fig. 9). The top three biological functions associated with this network are gene expression, cell-to-cell signaling (i.e. between blastomeres), and cellular movement, the latter two being essential to blastocyst formation and ultimately interaction between the early embryo and the uterine epithelium. It is also noteworthy that all candidates except one were over-expressed *in vitro* compared with *in vivo*, indicating that *in vitro* embryos may indeed be metabolically more active.

Discussion

IVP of bovine embryos is practiced worldwide both for research and for breeding purposes in the dairy industry. The commercial context of bovine embryo production has led to the production of records that have made it possible to study the potential impact of this assisted reproductive technology (van Wagtenonk-de Leeuw *et al.* 2000). Short-term effects such as higher or lower cell number (Fischer-Brown *et al.* 2002), altered sex ratio (Kimura *et al.* 2005, 2008), changes in developmental kinetics (Holm *et al.* 2002), and lower tolerance to cryopreservation (Rizos *et al.* 2003) and long-term effects such as large offspring syndrome (Young *et al.* 1998) and increased frequencies of abnormalities and spontaneous abortion (Taverne *et al.* 2002) are well documented. The mechanistic causes of

Figure 5 Clustering of co-regulated genes for the various embryo production systems. Heat map from the 100 candidates with the most significant *P* values. Reference to treatment number presented in Fig. 1 is indicated.

these phenotypes have not yet been elucidated. It is widely accepted that although bovine blastocysts can be produced under a wide range of *in vitro* conditions with some success, IVP in its present state of development clearly provides suboptimal conditions. So far, the most common basis of comparison between IVP systems remains the percentage of zygotes reaching the blastocyst stage (Menck *et al.* 1997, de Moraes & Hansen 1997, Guyader-Joly *et al.* 1999, Luciano *et al.* 1999, Stojkovic *et al.* 2002, Alm *et al.* 2005, Fujita *et al.* 2006, Block *et al.* 2009, Pereira *et al.* 2010). However, blastocyst yield might not be an appropriate measurement of IVP efficiency if embryo quality is defined as the potential for healthy gestation and successful coping with stresses such as cryopreservation and transfer protocols. Indeed, molecular study on the responses of embryos to different conditions would greatly improve our understanding of early embryo development and no doubt lead to the development of highly efficient culture systems. In recent years, gene expression analysis has proven to be a powerful tool for such evaluation and its application to studying the effects of IVC media on bovine embryos appears timely.

Although many comparisons of *in vivo* and *in vitro* embryos have been published, the lack of uniformity among the methods used impedes the integration of this information into a much-needed overall appreciation of the concept of embryo developmental plasticity. The relative impacts of the different IVP systems and the extent to which these systems produce comparable embryos have not been adequately described. Although a certain level of developmental plasticity is expected, the degree of similarity between blastocysts produced using different IVP systems is not known. As anticipated, significant differences between the blastocyst yields of the ten IVP systems were observed on day 7.5 after fertilization. By combining blastocyst yield information, the overall appearance of the embryos and comparison of gene expression profiles, we seek to elucidate the similarities and differences between blastocysts produced using these various IVP systems and determine the conditions that yield embryos most similar to their *in vivo* counterparts. Overall, the observed yields and phenotypes for the standard IVP systems routinely employed worldwide (treatments 2, 3, 4, 5, 8, 9, and 10) were within expectations. However, one of the experimental treatments (6: SOF-BSA/SOF-BSA 5 days + SOF-serum 2 days) yielded fewer blastocysts, although the overall gene expression of the resulting embryos matched that of *in vivo* embryos more closely. Meanwhile, good blastocyst yields in some cases led (e.g. treatments 4 and 5) to the greatest divergence between replicates and the farthest clustering from the *in vivo* reference, suggesting that adding serum to the IVC medium results in a more heterogeneous blastocyst population. These observations may be due to stimulation and survival of embryos that otherwise would have died.

Figure 6 Influence of IVP conditions on abundance measurements of long non-coding RNA and mitochondrial transcripts. (A) qRT-PCR measurements across different SOF-based *in vitro* systems. The IVM and IVC conditions are indicated for all *in vitro* systems. *In vivo* embryos were included as a reference treatment for most targets. Chr25-10.57M, Chr25-4.79M, and ChrX-26.5M are nuclear lncRNA, whereas ND5 and 12S are mitochondrial targets. Reference to treatment number presented in Fig. 1 is indicated. (B) Localization of the expressed sequence tag on the nuclear and mitochondrial genome. The bright red arrow indicates the mitochondrial expressed sequence tags found on the microarray.

Taken together, these results bring a novel perspective to the quest to define high-yielding IVP systems where the use of these suboptimal conditions can act as a mean to select the most robust embryos that exhibit the best developmental competence. In a situation where the exposure to such suboptimal conditions does not lead to long-term dire effects, this selection and homogenization of the embryonic cohort could be beneficial in maximizing the transfer of the best embryos. Embryo quality remains a vague concept, but these results suggest that the combination of morphological characteristics with overall gene expression profiling could provide more efficient means of describing and distinguishing the functional characteristics of groups of embryos.

At the gene level, relative transcript abundance analysis has proven to be challenging. Although a daunting task, associating fluctuations in gene expression with deleterious phenotypes has considerable potential for identifying faulty gene pathways and suggesting improvements to culture conditions. We have previously found that the comparison of gene lists is another endeavor hindered by methodological considerations. In benchmarking our own microarray platform, we recently reported the major impact of sample processing procedures on gene expression results (Gilbert *et al.* 2009, 2010). For instance, the correlation values between different sample amplification procedures, which are inherent when working with early embryos, were in the range of 0.35 (Gilbert *et al.* 2010). By generating our dataset from a single standardized methodological platform, we hoped to ensure the validity of all of the comparisons made.

One of the specific aims of this study was to determine the impact of serum supplementation on gene expression in embryos cultured in IVM and IVC media. We note with interest that among the biological functions differentially affected between these two phases were general cellular functions and also functions (e.g. lipid metabolism) that could be associated with observable phenotypes. It has been clearly shown that *in vitro* produced embryos are generally darker due to lipid over-accumulation, which translates into a lower tolerance to cryopreservation (Crosier *et al.* 2000, Rizos *et al.* 2003). Furthermore, in contrasting the *in vitro* treatments with the *in vivo* reference, only one gene network clearly emerged as being involved in the phenotypes displayed by IVP embryos even though several gene networks were expected. Although the biological functions associated with this network are broad, the fact that all of its genes (with the exception of peptidylprolyl isomerase A or PPIA, also known as cyclophilin A) were over-expressed in *in vitro* embryos could be related to the 'quiet embryo' theory (Leese 2002, Leese *et al.* 2007). It has recently been proposed that the best embryos would be those with the lowest metabolism and thus overall lower level of gene expression. Leese *et al.* (2008) propose two

Figure 7 Pathway influenced by the presence of serum during either IVM or IVC (treatments 3 and 4). Data was generated by contrasting treatments 3 and 4 (SOF-serum/SOF-BSA versus SOF-BSA/SOF-serum). Red = over-expressed when serum is added during oocyte maturation; green = over-expressed when serum is added during embryo culture.

categories of 'quietness' within a cohort of embryos: the metabolically less active condition being more viable (inter-individual quietness) and 'loss of quietness' occurring in response to stress such as the culture environment. This hypothesis is still debated and it is not clear why a higher metabolic state confers lower developmental potential rather than increasing means of coping and compensating.

Our results clearly indicate that culture conditions not only impact protein-coding transcripts but also non-coding RNA in both the nuclear and the mitochondrial genome. In fact, the most compelling attribute of the clone collection featured on the 'boutique' array used in this study is the presence of uncharacterized transcripts ($n=161$). By assembling a clone collection without relying on *a priori* knowledge, we were able to isolate a set of NTR, of which the abundance is impacted by embryo production systems. The roles of these long uncharacterized transcripts still remain to be elucidated. All of the novel candidates confirmed as being influenced by culture conditions are presumed to be long non-coding RNA (lncRNA) because they are located in intergenic regions in which the known flanking genes were found to be several dozen kb away. It is highly probable that the clones assembled for printing on the microarray are partial in the case of these novel sequences. The full transcript structure is unknown but the cloned sequences did not harbor any open

reading frame that would be indicative of a protein-encoding transcript.

The cellular functions of lncRNA are still unclear, but some of the best-studied examples such as H19 and the X-specific inactivating transcript (Xist) have been shown to play crucial roles during early development (Rougeulle & Avner 2004, Senner & Brockdorff 2009). The action of these lncRNA, along with small non-coding RNA (which include micro-RNA), is part of the epigenomic regulation of gene expression. The finding that these lncRNA are influenced by culture conditions clearly highlights the level of complexity inherent in early development. It is still unknown whether these lncRNA are indicative of faulty chromatin structure rendering their expression permissive, which would corroborate reports suggesting that culture conditions and embryonic manipulation can perturb the epigenome (Le Bouc *et al.* 2010, Santos *et al.* 2010), or if they are involved in a deliberate response that enables the embryo to cope with culture conditions. Genomic assembly of these ESTs and functional analyses through their knock down is underway. Our results showing that, in addition to protein encoding RNAs, mitochondrial RNAs and also lncRNAs are important component of the embryonic transcriptome corroborates results from Bui *et al.* (2009). However, in this report, quantification of one of the retrotransposons at the morulae stage showed similar levels between *in vitro* fertilized and

Figure 8 List of biological functions impacted by serum supplementation either during IVM or IVC (contrasting treatments 3 and 4). Significance threshold is indicated.

cloned from somatic cell nuclear transfer (SCNT) counterparts, which is indicative of normal reprogramming (Bui *et al.* 2009). In contrast, we report in this study that the *in vitro* culture environment is influencing the level of expression of lncRNAs. It could then be expected that increased embryonic manipulations such as performed on SCNT embryos would lead to aberrant expression of retrotransposons. The fact that both embryonic types were submitted to the same IVP system and both involved selection of morphologically similar embryos may explain this apparent discrepancy between studies. A better understanding of the potential roles of these lncRNAs during early development most certainly requires further investigation.

Conclusion

The dataset generated in this study enabled to perform comparisons that provided more information about IVP systems than does standard determination of developmental rate alone. They also provided details that are complementary to work already published and suggest additional parameters for defining the concept of embryo quality, such as cohort homogeneity. Finally, to our knowledge, this is the first study reporting that the expression of novel lncRNA is influenced by IVP conditions.

Materials and Methods

All chemicals were obtained from Sigma–Aldrich unless stated.

Embryo production

Production of *in vivo* blastocysts

The ovarian stimulation protocol, artificial stimulation, and uterine flushing were conducted as described by Bousquet *et al.* (1999).

Oocyte maturation

Ovaries from dairy cows were collected at the slaughterhouse and processed as described previously. Cumulus oocyte complexes (COCs) were washed in HEPES-buffered Tyrode's lactate (TLH) medium supplemented with 10% (V/V) FCS and placed in groups of ten in pre-equilibrated 50 µl droplets of oocyte maturation medium (see below) covered by mineral oil. After the distribution of the COCs, the drops were topped with 0.1 µg/ml FSH (Gonal-f, Serono) and 1 µl/ml estradiol (Sigma #E2758). The following oocyte maturation media were used:

1. TCM (Invitrogen #11150) supplemented with 10% (v/v) FCS (or 'serum').
2. SOF (Holm *et al.* 1999, Vigneault *et al.* 2009) supplemented with 10% (v/v) FCS.
3. SOF supplemented with 0.8% BSA fraction V (Sigma).
4. SOF without protein supplementation.

Oocyte maturation was conducted for 24 h at 38.5 °C under a humidified atmosphere stabilized at 5% CO₂.

IVF

COCs were recovered from the maturation droplets and washed twice in TLH medium containing 3 mg/ml fatty acid-free BSA. An additional wash was performed in equilibrated IVF medium (Tyrode's medium supplemented with 6 mg/ml fatty acid-free BSA). COCs were then transferred to the fertilization chamber in groups of five, each in a 48 µl droplet of IVF medium. On transfer, each droplet was supplemented with 2 µl of a solution containing 2 mM penicillamine (cat # P4875), 1 mM hypotaurine (cat # PH1384), and 250 µM epinephrine (cat # E4250).

Semen was a gift from the Centre d'insémination artificielle du Québec (CIAQ). It consisted of a pool of ejaculates from five different bulls. Standard fertility parameters were assessed according to commercial standards and semen was prepared for IVF as described previously to obtain a final concentration of 25×10⁶ spermatozoa per ml, of which 2 µl was added to each COC-containing IVF droplet. IVF took place in an incubator (5% CO₂) for 18 h at 38.5 °C.

In vitro embryo development

The presumed zygotes were collected and transferred to Eppendorf tubes containing TLH supplemented with BSA and gentamicin. Cumulus cells were then stripped from the zygote by repeated pipetting. The denuded zygotes were washed in Ca²⁺/Mg²⁺-free PBS supplemented with 0.3% BSA and gentamicin. Embryos were then distributed in culture droplets of the following composition:

1. SOF with 0.8% fatty acid-free BSA.
2. SOF supplemented with 10% (v/v) FCS.

Figure 9 Pathway influenced by the *in vitro* conditions across all tested systems. Data were generated from the contrast of all *in vitro* systems (treatments 2–11) with the *in vivo* reference (treatment 1). Red=over-expressed in *in vivo* blastocysts; green=over-expressed in *in vitro* embryos.

3. SOF without BSA or FCS (without protein supplementation).
4. TCM supplemented with 10% (v/v) FCS.
5. SOF with 0.8% fatty acid-free BSA for the first 5 days of culture followed by a transfer in droplets containing SOF supplemented with 10% (v/v) FCS for the last 2 days of culture.
6. Co-cultured (SOF or TCM) with BRL cells (co-culture) (with 10% (v/v) FCS).

Embryo development was conducted in a controlled atmosphere (5% CO₂, 7% O₂, and maximum humidity at 38.5 °C). Blastocysts were collected 7.5 days after fertilization and pooled in groups of ten. Samples were frozen and kept at –80 °C until RNA extraction.

Microscopy

Images of blastocyst groups were taken using a Zeiss stereomicroscope with a 40× objective. The images were captured using a digital camera mounted on the microscope.

Experimental design

A summary of all of the treatments is shown in Fig. 1. Blastocysts obtained *in vivo* were used as the reference in all comparisons with IVP systems. For all ten *in vitro* systems, oocyte fertilization was conducted using the same procedure (medium, semen source, and preparation). Oocyte maturation and embryo development were conducted using combinations of two culture media (TCM or SOF), supplemented with either BSA or FCS. Some production systems included the culture of embryos with the support of feeder cells (co-culture). A production system (SOF/SOF) was used as a suboptimal control condition.

For this system, blastocysts were produced in SOF medium without any protein supplementation. For each production system, three biological replicates were done. Technical duplicates for each of these 33 samples were analyzed to generate a dataset composed of 66 microarray hybridizations. The microarray dataset is publicly available at the GEO Omnibus repository (GEO accession number: GSE27872).

Description of the microarray

The BlueChip microarray version 1.2 (Vallee *et al.* 2009) was used in this study. This microarray contains cDNAs collected from four subtracted libraries (two libraries for isolating transcripts prevalent in oocytes or blastocysts by using a pool of somatic cells as driver while the other two libraries compared oocytes and blastocysts together on the forward and reverse subtractions) generated as published previously (Robert *et al.* 2000). Each microarray contains a total of 1232 features, each spotted twice. Among these features, 1066 are unique (740 candidates with a RefSeq ID, 161 novel transcripts, 129 not sequenced, and 36 uncharacterized loci) and 166 are controls (negative: two types of Spot Report Alien cDNA Array Validation System (Stratagene, La Jolla, CA, USA, *n*=16); H₂O/DMSO (*n*=50); GFP (*n*=28); arabidopsis (*n*=8); empty (*n*=32); positive: ubiquitin (*n*=8); α -tubulin (*n*=8); β -actin (*n*=8)).

Microarray experiments

RNA extraction

Total RNA (from pools of ten blastocysts) was extracted using PicoPure columns (Molecular Devices, Sunnyvale, CA, USA) following the manufacturer's instructions. A DNase (Qiagen)

Table 2 PCR primer sequences and reaction conditions.

Gene name	Gene symbol	Primer sequences 5'–3'	Amplicon size (bp)	Annealing/ acquisition temperature (°C)	Accession number
Placenta-specific 8	<i>PLAC8</i>	F: TCGCCATGAGGACAATGTATCGGA R: GCTTGAGTTGACAAAGGGCACAGA	108	50/80	BC114195
Lactate dehydrogenase B	<i>LDHB</i>	F: CAGTGTCAACAATGGTGAAGGGCA R: GCAAGCATTAAAGCAAGCATGGGC	346	60/82	BC102217
Plasminogen activator, urokinase	<i>PLAU</i>	F: AGTCTGGTTTCCCATGAGGTGTGT R: TGAGTGTGATCCAGGGCAGGAAT	254	60/86	BC122657
3-Hydroxy-3-methylglutaryl-CoA synthase 1	<i>HMGCS1</i>	F: CAAGCCCTGCTAAGAAAGTGCCAA R: TGAAGTCTCCACCTCACAGAAGT	110	59/80	AY581197
Ribosomal protein S4, X-linked	<i>RPS4X</i>	F: CCATTCAGATTGACTTGGAGACTGGC R: AGAGAGATCCACGGTTTGTTCCT	238	60/80	XM_001249535
Mitochondrially encoded NADH dehydrogenase 5	<i>ND5</i>	F: CGAAAACAACCCCTTCTG R: AGGCGTTTGAGGGGTAGTG	227	50/78	NC_006853
12S rRNA	<i>12S</i>	F: GCAAGCATCTACACCCAGT R: GCTGGCACGAGATTTACCA	195	58/83	NC_006853
Novel transcribed region	<i>Chr25-4.79M</i>	F: GGTCTGAACAGCGTGTGT R: TGATGTGCAGAGGCAACA	248	58/85	NA
Novel transcribed region	<i>Chr25-10.57M</i>	F: GGATCGCAAGACGTACTCC R: CAAAGTCCAAGGAGGTTCA	243	56/78	NA
Novel transcribed region	<i>ChrX-26.5M</i>	F: CAGAATCGCATGGTTGAGTG R: TGCAGGAGGTGTTTCCAAG	213	56/76	NA
Novel transcribed region	Unassigned	F: GTAGGACTTGTGCTAGGGCG R: CATGTGGCTCCTAGCCTGAG	246	55/83	NA
β-Actin	<i>ACTB</i>	F: ATCGTCCACCGCAAATGCTTCT R: GCCATGCCAATCTCATCTCGTT	101	59/80	NM_173979
Conserved helix–loop–helix ubiquitous kinase	<i>CHUK</i>	F: TGATGGAATCTCTGGAACAGCG R: TGCTTACAGCCCAACAATTGC	180	57/81	NM_174021
Green fluorescent protein	<i>GFP</i>	F: GCAGAAGAACGGCATCAAGGTGAA R: TGGGTGCTCAGGTAGTGGTTGT	143	59/89	NA
Glyceraldehyde-3-phosphate dehydrogenase	<i>GAPDH</i>	F: CCAACGTGTCTGTTGTGGATCTGA R: GAGCTTGACAAAGTGGTCGTTGAG	217	58/84	NM_001034034
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	<i>YWHAZ</i>	F: GCAGAAGAACGGCATCAAGGTGAA R: CTTACAGTTCGTCCTCTGG	286	55/79	NM_174814

treatment was performed on column. RNA samples were amplified through two rounds of *in vitro* transcription (IVT) using the RiboAmp amplification method (Molecular Devices) according to the manufacturer's recommendation with one modification. During the second amplification round, amino-allyl-coupled nucleotides were added for labeling purposes. During this second amplification reaction, the IVT Master Mix was replaced with the amino-allyl mix (Molecular Devices). Microarray hybridization samples consisted of 5 µg amino-allyl-containing aRNA labeled with Alexa 647 dye (Invitrogen). Chemical bonding of the dye to amino-allyl groups was performed following the manufacturer's instructions. Unincorporated dye molecules were removed by purifying samples with the RNA Clean Up RNeasy Minikit (Qiagen). Samples were concentrated by isopropanol precipitation and pellets were resuspended in 5 µl RNase-free water (Ambion, Austin, TX, USA).

Microarray hybridization and data acquisition

The concentrated labeled samples were mixed with 80 µl hybridization buffer (HybBuffer #1 from Ambion) and deposited on the microarray under a LifterSlip coverslip (Thermo Scientific, Mississauga, ON, Canada). Microarray

incubation was conducted for 21 h at 50 °C in an automated station (the Slidebooster apparatus from Advantix, Danvers, MA, USA).

The coverslips were then gently removed in a low-stringency buffer (2 × SSC + 0.5 × SDS). Slides were washed twice in the low-stringency buffer for 15 min at 50 °C, transferred to high-stringency buffer (0.5 × SSC + 0.5 × SDS), and washed twice for 15 min at 50 °C and finally dipped quickly in a 1.25 × SSC solution and dried by centrifugation at 1200 *g* for 5 min at room temperature. Slides were read on a VersArray ChipReader (Bio-Rad). Image analysis was performed using ArrayPro software version 4.5 (MediaCybernetics, Bethesda, MD, USA). For each spot, background was determined locally. Data tables containing the foreground, background, and flagged spot annotation were generated for downstream data processing and statistical analyses.

Quantitative RT-PCR measurements

Additional pools of ten blastocysts per sample were collected for several treatments. Total RNA was extracted as described above. RT was conducted according to the manufacturer's recommendations using the qScript cDNA SuperMix (Quanta

Biosciences, Gaithersburg, MD, USA) with an oligo-dT to prime the reaction.

Primers for each candidate were designed using the Primer3 Web interface (<http://frodo.wi.mit.edu/primer3/>). Primer sequence, annealing and fluorescence acquisition temperatures, amplicon size and GenBank accession numbers are shown in Table 2. The reaction mixture was composed of the LightCycler FastStart DNA Master SYBR Green I kit components (Roche) and real-time measurements were conducted in a LightCycler 2.0 apparatus (Roche). Our real-time PCR amplification procedure has previously been described in detail (Gilbert *et al.* 2010). The nature of the amplified products was confirmed using the melting curve profile and DNA sequencing.

Data analysis

Microarray data were pre-processed as follows: 1) background correction was conducted by simple subtraction; 2) mean values were calculated for the technically duplicated arrays; 3) median values were calculated for technically duplicated features spotted on the microarrays; 4) within-array normalization was performed with loess; 5) quantile was applied for inter-array normalization; and 6) the entire dataset was trimmed according to a cut-off value calculated from the mean values of selected negative control features (aliens, GFP, and arabidopsis) plus two s.d. Steps 3–5 as well as the statistical analysis and all downstream steps were conducted in WebArrayDB (Xia *et al.* 2005, Wang *et al.* 2009): <http://www.webarraydb.org/webarray/index.html>.

The normalized and trimmed dataset was subjected to significance testing using the LIMMA algorithm (Smyth 2004, Smyth *et al.* 2005) using one of the treatments (or a group of treatments) as the reference. For contrasts set across all treatments, *in vivo* produced embryos were set as the reference. Adjusted *P* values were calculated using the standard Benjamini–Hochberg method (Hochberg & Benjamini 1990). Hierarchical clustering, heat maps, and between-group analyses (Culhane *et al.* 2002, 2003) were performed in WebArrayDB.

Pathway analyses and downstream exploitation of gene lists were conducted using Ingenuity Pathway Analysis Software Version 8.6 (Ingenuity Systems, Inc., Redwood City, CA, USA). For biological functions and canonical pathways, significance and threshold values were calculated using Fisher's exact test ($P < 0.05$). Only functions that included at least four candidates were selected Fig. 6, while a minimum of two genes was required for canonical pathways (Fig. 8).

For qRT-PCR data, normalization was performed using the geometric mean of several housekeeping candidates as proposed by Goossens *et al.* (2005). Calculations were performed using the geNorm Web platform (Vandesompele *et al.* 2002; <http://medgen.ugent.be/~jvdesomp/genorm/>). The housekeeping candidates included conserved helix–loop–helix ubiquitous kinase, glyceraldehyde-3-phosphate dehydrogenase, β -actin, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide in addition to a spiked in control mRNA bearing a poly(A) tail (GFP transcript). Testing of statistical significance of observed differences was done using Prism software Version 5.0

(GraphPad Software, La Jolla, CA, USA). One-way ANOVA performed done using Kruskal–Wallis and Dunn's multiple comparison tests.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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