What have we learned from the embryonic transcriptome?

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Abstract

During the last decade, transcriptome profiling has emerged as an efficient approach to describe and study cellular functions. The potential to survey transcript abundance for all genes offers promise to shed light on mammalian early embryogenesis. Furthermore, the report of aberrant phenotypes following the application of reproductive technologies also fueled the need to understand how embryos react, cope and adapt to their surrounding microenvironment. So far, the atypical nature of early blastomeres and the drastic transitions through which embryogenesis progresses posed and still pose numerous technical challenges such as to correctly interpret the natural fluctuation in total RNA and proteins contents. Although tedious, these technical considerations are important for data soundness and interpretation. In this review, we examine a number of transcriptomic surveys performed on blastocysts and demonstrate that several consistent observations have transpired that alter the conceptual issues regarding the definition of embryonic normalcy. Moreover, the need to complement the study of gene expression with profiling epigenomic marks is opening new perspectives that will also be discussed.

Introduction

Regulation and modification of gene expression are what allow every living being to respond to varying cellular needs and to adapt to a changing environment. Modification is observable mainly in terms of gene transcription products (i.e. messenger RNA) and translation products (i.e. proteins). It is therefore expected that the study of gene product expression level will be indicative of physiological state. The roles of transcriptional regulators including factors acting on gene promoters and their complement of gene enhancer and silencer elements in driving expression have been known for decades. An additional level of complexity identified more recently is known as epigenetics: modification of gene expression driven by dynamic chromatin configurations and a multitude of nuclear phenomena. Gene expression and its regulation have become the focus of many studies, and are at the core of our efforts to understand early development, that is, the makeup, physiology and outcome of the early embryo.

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In the early 2000s, advances in genomics and bioinformatics provided the opportunity to apply a more comprehensive methodology than conventional gene candidate approaches. The term ¨transcriptomics¨ was coined as a reference to methodologies capable to surveying the transcript population with a scope well superior than what is offered by RT-qPCR. The current definition of transcriptomics does not reach consensus where a restrictive view is limiting the term to samples processed on Next Generation Sequencing platforms that surveys most transcripts (a selection of transcripts based on size and/or presence of poly(A) tail is generally performed during library preparation) while a more lenient acceptance in term usage includes large but partial surveys. Here transcriptomics refers to the study of total or partial set of transcripts from a tissue or a cell population. In fact, the development of high-throughput platforms for the measurement of gene expression has caused a paradigm shift in the enunciation of initial hypotheses, broadening the initial premises to include the concept that differences in physiological state among experimental specimens are reflected in gene product abundance (primarily mRNA) and ultimately the molecular pathways being regulated through gene expression. In other words, by observing deviations in RNA abundance, we can identify the physiological processes underlying phenotype or developmental outcome.

The first high-throughput platforms dedicated to the survey of mRNA were cDNA microarrays developed and used successfully to estimate the transcriptional contents of early embryos (Ko *et al*. 2000, Tanaka *et al*. 2000, Carter *et al*. 2003, Sirard *et al*. 2005, Misirlioglu *et al*. 2006, Somers *et al*. 2006). The study of early mammalian development always has been and remains technically challenging, since embryos are a scant source of biological material. However, unlike protein profiles, the transcriptome can be amplified starting from minute amounts of material, and this approach has gained wide popularity and is now routine practice. The challenges of limited sample size have thus been met for transcriptome profiling and science is now turning its attention to single-cell transcriptome analysis (Tang *et al*. 2011, Katayama *et al*. 2013).

It is becoming clear that as technical challenges are overcome and the more we analyse cellular gene expression, the more complex developmental physiology seems to be. For instance, while microarrays have provided means of interrogating all known genes in a sample, thus raising the possibility of unexpected findings such as the involvement of gene pathways, not previously known to be active during early development, they are now criticized for their limitation of addressing only the transcript fragments corresponding to the oligo/probe set. This criticism has been addressed with the advent of next-generation sequencing (RNA-seq), which facilitates RNA sequencing, thus broadening the scope of the survey to include populations such as mRNA, miRNA, tRNA, LncRNA andrRNA (Wilhelm *et al*. 2008) Marioni *et al*. 2008.

Through Next-Generation RNA sequencing (RNA-seq), the complexity of the transcriptome has become obvious, since in addition to differential gene expression intensities, higher level of complex features such as multiple splicing variants within or outside the protein-coding region and an entire population of novel transcripts with yet unknown functions have emerged. We have only begun to scratch the surface of the potential implications of alternative splicing for protein diversity, of the stabilization and destabilization of RNA, or of these uncharacterized transcripts as modulators of embryonic phenotypes.

The aim of this review is to provide a current perspective on the strides that have been made in transcriptomic studies of mammalian embryos. The challenge to integrate transcriptomics information into a physiological context will also be discussed.

RNA abundance in early development and its correlation with physiological functions

One of the first observations made when studying RNA management in early blastomeres is that gene regulation falls outside of the textbook model in which expression relies on synthesis

of pre-mRNA transcripts that are spliced, capped and polyadenylated before transport from the nucleus to the rough endoplasmic reticulum, where ribosomes translate the functional mRNA to polypeptides that are subsequently processed in the cytoplasm. Based on this standard chain of events, protein production is expected to reflect cell mRNA content. However, oocytes and the blastomeres rely on maternal RNA reserves accumulated during oogenesis to sustain protein synthesis, especially during the first cell divisions, which occur while nuclear transcription is minimal, if not absent (Plante *et al.* 1994, Memili *et al.* 1998, Lodde *et al.* 2008). This initial period of transcriptional silence is species-specific (Braude *et al.* 1988, Poueymirou & Schultz 1989, Memili *et al.* 1998). It is believed that a large proportion of the maternal RNA reserves are never translated and is instead simply decomposed (Bachvarova & De Leon 1980, Su *et al.* 2007, Ma *et al.* 2013). The explanation for such apparent wastage of a major expenditure of energy and immobilization of material resources is unknown. In mice, destruction of maternal RNA is a an essential prelude to embryonic genome activation (Ma *et al.* 2013). In non-mammalian model organisms, degradation of maternal RNA has been shown to start with binding of microRNA to specific sequences in the 3′ untranslated region during pre-embryonic genome activation (Tadros & Lipshitz 2005, Giraldez *et al.* 2006, Lund *et al.* 2009). In large mammals, embryonic genome activation occurs several cell cycles later than in mice, maternal RNA stores are utilized to sustain protein synthesis during this extended period of transcriptional silence, and maternal reserves are used and degraded more gradually (Gilbert *et al.* 2009). It remains very difficult to draw any conclusion regarding the biological implications of transcript abundance where endogenous reserves and the possibility of RNA degradation prior to translation are involved (Gilbert *et al.* 2009).

Mammalian somatic cells generally contain 20–30 pg of total RNA, of which 1–5 % is mRNA. In the case of oocytes and early blastomeres, these figures are very different: A bovine fully grown GV stage oocyte contains around 340 pg of total RNA with a proportion of 18% of mRNA whereas a bovine blastocyst contains 2,350 pg of total RNA (Figure 1) (Gilbert *et al.* 2009). Oocytes accumulate RNA reserves during their growth and these maternal stocks are used to sustain protein synthesis until embryonic genome activation (Clegg & Piko 1982, Piko & Clegg 1982, Bachvarova *et al.* 1985, Paris & Richter 1990, Paynton & Bachvarova 1994). This implies that cells contain large quantities of dormant RNA and that transcript abundance may not be indicative of protein synthesis. This is true for the entire pre-embryonic genome activation period, after which gene expression becomes more like in somatic cells. When RNA reserves are present, no scenario can be excluded: transcripts and their corresponding proteins may both be abundant, or transcripts may be abundant while the proteins are completely absent (Pennetier *et al.* 2004, Vigneault *et al.* 2009). In addition, in embryos in which genome activation occurs days after fertilization (i.e. in large mammals), ribosomal RNA profiles are very different from those of somatic cells. Microelectrophoretic profiles of total RNA obtained prior to embryonic genome activation show the two large ribosomal RNA molecules present in ratios far from the 18S: 28S theoretical maximum of 2.5 used as an indicator of sample quality in somatic cells (Gilbert *et al.* 2009). The physiological implications of this atypical ribosomal RNA content are still unclear. During the stages prior to embryonic genome activation, 28S RNA is detected in low amounts, and considering that it is the major component of the 60S ribosomal subunit, this may influence cell capacity for translation and/or ribosome turnover rate (Gilbert *et al.* 2009).

It is still unclear how dormant maternal RNA is recruited and sent either for protein synthesis or for breakdown. Clear evidence of the "closed-loop" model proposed in *Xenopus* and involving cytoplasmic polyadenylation-element-binding protein and Maskin to control RNA recruitment and translation (McGrew & Richter 1990, Kim & Richter 2008, Martineau *et al.* 2008, Radford *et al.* 2008) has not been shown in mammals. Differences could exist between the strategies

that amphibians and mammals devised as they evolved to manage maternal RNA. In *Xenopus*, embryonic genome activation occurs when the number of cells reaches 4,000–8,000, which corresponds to 12–13 divisions and occurs within seven hours (Newport & Kirschner 1982b, Newport & Kirschner 1982a). For such demanding and rapid recruitment of maternal RNA, mechanisms of storage may differ from those of mammals.

Conserved mechanisms of maternal RNA storage include poly(A) tail removal and re-elongation, the former rendering the molecule dormant and the latter required for recruitment. However, stored RNA are not completely deadenylated and variable length in poly(A) tails is observed (Gray & Wickens 1998, Gohin *et al.* 2014). Prior to embryonic genome activation, the transcript population is therefore composed of molecules bearing a very wide range of poly(A) tail lengths (Lequarre *et al.* 2004). Figure 1 provides an overview of the levels of RNA found in this developmental window. Oligo-dT may be used during sample processing to select different subpopulations of transcripts (Robert 2010). Since protein synthesis requires transcripts bearing a poly(A) tail, this use of oligo-dT might introduce a bias against stored transcripts. Whether or not this occurs is uncertain since the extent of tail shortening and lengthening required for RNA storage and recruitment is unknown and clear segregation based on tail length might not exist. Data need to be interpreted with caution since any comparison of treatments showing lower abundance of RNA during the maternal stage could be attributed to loss due to protein synthesis, to different poly(A) tail lengths or to increased rates of decay without translation.

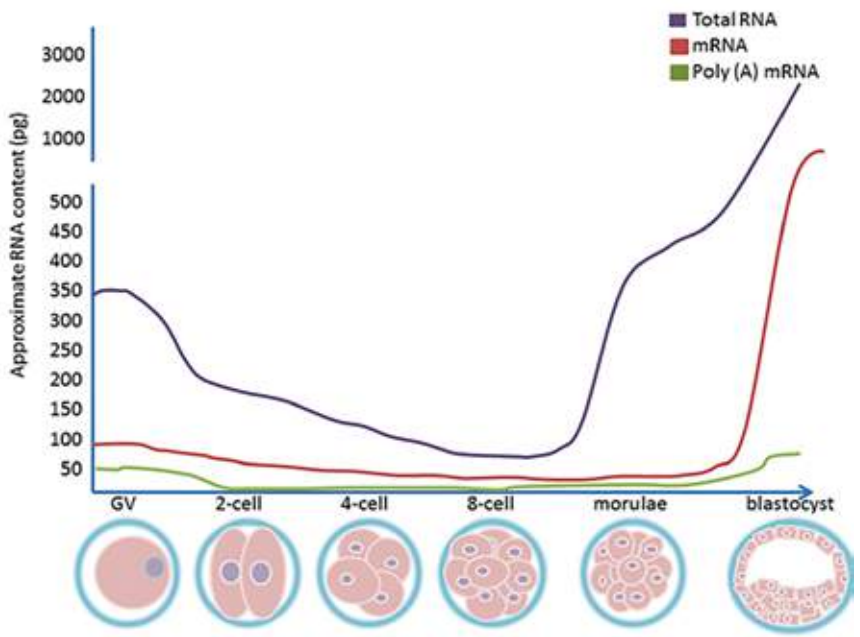

Figure 1. Schematic illustration of the different categories of RNA found in bovine oocytes and in embryos during the early stages of development

Even more challenging than interpreting transcript abundance prior to embryonic genome activation (no matter the technological platform) is the comparison of transcript abundance across developmental stages. Since each developmental stage differs in terms of cell number and size and RNA and protein content, there are no points of reference to which data can be easily interpreted in its physiological context. Prior to embryonic genome activation, cell number is irrelevant since the RNA content of a blastomere represents a subdivision of the

maternal RNA pool present at the previous stage. Following genome activation, cell number matters but it is still difficult to abandon the concept of the embryo as the biological unit and switch to accounting for cell numbers, because of a lack of reference for the interpretation of transcript level per blastomere.

The dynamics of early embryogenesis pose many challenges for RNA profiling and data interpretation (Figure 2). Sample preparation methodology and data normalization can influence measurements and data interpretation profoundly (Gilbert *et al.* 2010). Over the years, spiking samples with exogenous transcripts has emerged as the most appropriate way to normalize datasets, since in a situation of overall reduction of cellular RNA content, all assumed housekeeping candidates will follow the same trend no matter how many reference genes are used, and thus contribute to data over-normalization (Robert 2010). A similar situation also occurs in high-throughput RNA profiling by microarrays and RNA sequencing (RNA-seq), since most normalization algorithms are based on leveling the mean or median between samples, which differ naturally throughout the various stages of development.

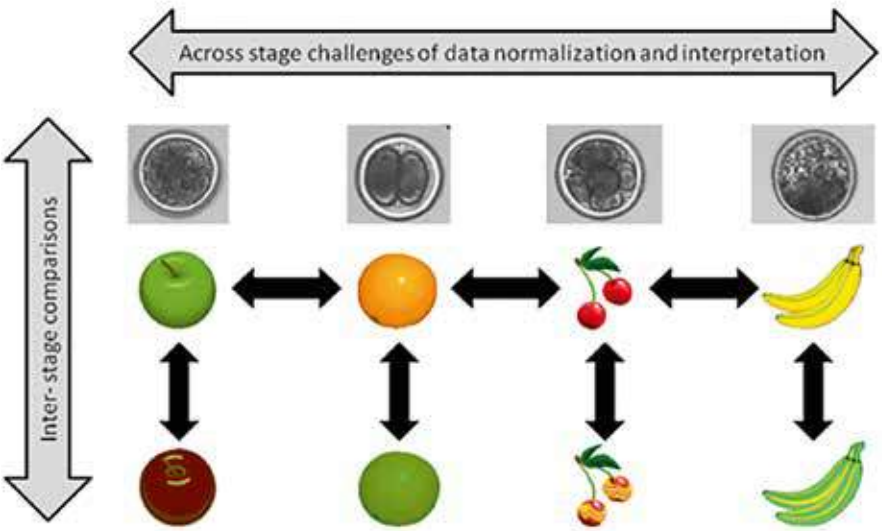

Figure 2. Different challenges arise when comparing samples within a developmental stage or across developmental stages. Due to large differences between embryos at different stages in terms of cell content (RNA, DNA, proteins, lipids, etc.), cell number and transcriptional potential, inter-stage comparison poses several challenges for data normalization and interpretation. In inter-stage comparisons, similar specimens will be compared, thus providing a more direct interpretation of treatment effects.

Elevated amounts of dormant RNA definitely hinder our attempts to describe mammalian early embryo physiology based on the transcriptome. It is well accepted that protein profiling would be a better-suited approach. However, overall profiling and identification of proteins still requires hundreds to thousands of oocytes or early embryos (Coenen *et al.* 2004, Massicotte *et al.* 2006, Berendt *et al.* 2009, Han *et al.* 2010). As an alternative, one efficient strategy for distinguishing between active and stored mRNA is to isolate subpopulations present in polyribosomal fractions (Potireddy *et al.* 2006, Chen *et al.* 2011, Scantland *et al.* 2011). Assuming that messenger RNA bound to polyribosomes is being processed for translation, it is presumed to be representative of gene activity and hence of the underlying physiological status. In mouse studies, comparison

of the GV stage to MII oocytes by polyribosomal fractionation has provided insight into the reduction of translation potential, while the comparison of zygotes and 2-cell embryos has provided information about the proteins required for embryonic genome activation (Potireddy *et al.* 2006, Chen *et al.* 2011).

The post-embryonic genome activation transcriptome

Most of the transcriptomic surveys carried out during pre-hatching development have been performed on blastocysts, since this is the last developmental stage that can be maintained in culture. So far, the main application for these RNA abundance measurements has been to describe the impact of assisted reproductive technologies on embryo physiology. In most studies, embryos obtained from oocytes fertilized *in vitro* have been compared to reference embryos collected *in vivo*. The need for better understanding of the impact of assisted reproductive technologies on early development grew from observations of greater prevalence of aberrant phenotypes in animals resulting from embryos produced *in vitro* or obtained from somatic cloning (Young *et al.* 1998, van Wagtendonk-de Leeuw *et al.* 2000, Gibbons *et al.* 2002, Smith *et al.* 2012), including enlarged placentas and fetuses in ruminants (Sinclair *et al.* 2000, De Sousa *et al.* 2001), abnormalities associated with increased prenatal loss and higher perinatal mortality, although these phenotypes may lead to perfectly sound offspring.

Earlier work aimed at identifying deviant gene expressions that could explain how early developmental stress translates into long-term consequences was conducted using RT-PCR (qualitative and quantitative) on candidate genes. Numerous studies have shown skewed responses to culture conditions. Impact on all types of molecular functions has been observed, including energetic metabolism, free radical management and synthesis of cell structural constituents (Wrenzycki *et al.* 1996, Wrenzycki *et al.* 1998, Niemann & Wrenzycki 2000, Wrenzycki *et al.* 2001, Yaseen *et al.* 2001, Lazzari *et al.* 2002, Wrenzycki *et al.* 2002, Lonergan *et al.* 2003a, Lonergan *et al.* 2003b, Lonergan *et al.* 2003c). Nowadays, the use of microarrays and RNA sequencing provides a more comprehensive picture of gene activity. It is now clear, based on observation of gene expression in a broad array of cell functions, that embryos react to perturbations in their microenvironment (Carter *et al.* 2010, Cote *et al.* 2011, Cagnone *et al.* 2012, Gad *et al.* 2012b, Plourde *et al.* 2012a, Plourde *et al.* 2012b, Cagnone & Sirard 2013, Cagnone & Sirard 2014). Gene expression overall tends to be more active in blastocysts of embryos produced *in vitro* than in their *in vivo* counterparts. This is consistent with the observation that metabolically quiet embryos tend to have better developmental potential (Leese 2002, Leese *et al.* 2007). Furthermore, *in vitro* conditions also influence the kinetics of embryo development (Mingoti *et al.* 2011), suggesting the possibility of stimulating or accelerating development, in vitro. Although perhaps unrelated physiologically, cleavage rate and developmental competence appear to be correlated (Lechniak *et al.* 2008, Isom *et al.* 2012, Orozco-Lucero *et al.* 2014). The transcriptomes of embryos produced in different culture media have been compared in several studies (Cote *et al.* 2011, Cagnone *et al.* 2012, Plourde *et al.* 2012b, Cagnone & Sirard 2013, Penagaricano *et al.* 2013, Cagnone & Sirard 2014). We have found in our laboratory that culture conditions providing poor developmental rates in some cases unexpectedly produced blastocysts with a transcriptome more similar to the *in vivo* reference than did conditions that yielded the highest blastocyst rates (Cote *et al.* 2011). Furthermore, a treatment intended as a negative control in which embryos were produced in media without serum, BSA or any macromolecule provided a low blastocyst rate as expected, but an overall transcriptome similar to that observed for the best-yielding system (Cote *et al.*

2011). In addition, low-yielding systems provided more consistent transcriptome profiles than did high-yield conditions (Cote *et al.* 2011). This is reflected in much greater morphological dispersion among embryo cohorts than is observed with high-yielding systems. Conceptual questions arose from these results (Figure 3): Does the stringency of low-yielding systems allow only highly competent embryos to survive while high-yielding systems keep compromised or deviant embryos alive? Would ill phenotypes be more frequent among blastocysts produced in high-yielding systems? Is optimization of *in vitro* conditions to obtain the highest blastocyst rates a justified goal? These questions remain unanswered.

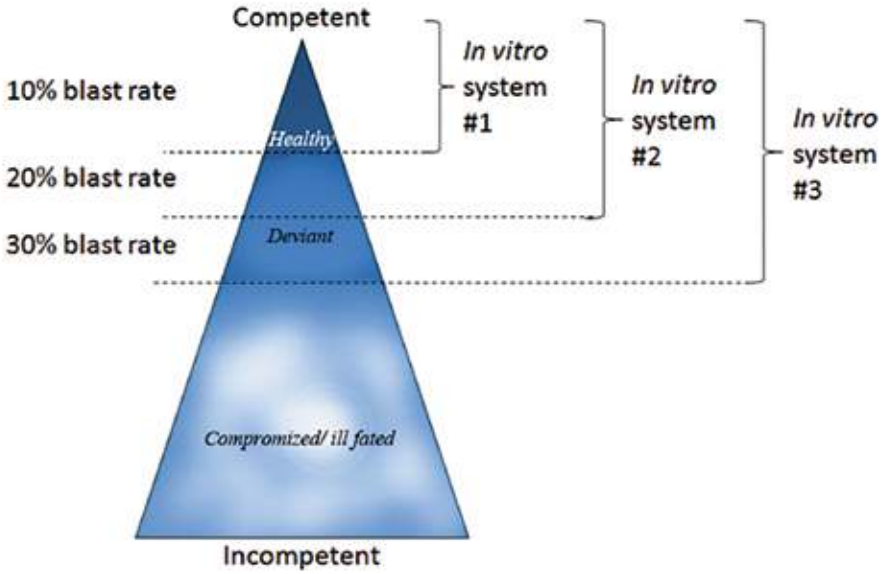

Figure 3. Intrinsic developmental potential is determined largely by the quality of the oocyte. Are observed differences between the performances of *in vitro* production systems merely indicative of stringency allowing only high-potential embryos to survive? Would the most competent oocytes reach the blastocyst stage in all systems or would the blastocysts yielded by different systems originate from oocytes with different characteristics?

It was also noted early in the study of the response of embryo transcriptomes to different assisted fertilization microenvironments that blastocyst cohort composition influenced the results. Some *in vitro* production systems produce cohorts that are more homogenous than others (Cote *et al.* 2011, Plourde *et al.* 2012b). In most studies, samples composed of a fixed number of pooled blastocysts are analysed. However, all blastocysts are not equal in terms of morphology and hence cell number, type (e.g. inner cell mass or trophectoderm) and viability. Comparing blastocysts of different morphological classes revealed important differences in transcriptome profile (Rekik *et al.* 2011). In addition, some culture conditions have been shown to skew the gender ratio and that male and female blastocysts exhibit large differences in gene expression (Bermejo-Alvarez *et al.* 2010, Garcia-Herreros *et al.* 2012). These observations presented a conundrum, since the most logical grouping of embryos for valid comparisons of *in vitro* treatments was according to morphology and gender. The treatment effects were thus confounded with effects due to these factors. Part of the solution to this problem would be to obtain transcriptome profiles of single blastocysts that had been sexed and morphologically classified. However, due to natural biological variance, this would require experimental designs with a larger number of biological replications in order to reach statistical significance, and

this for both genders and all morphological classes. This would allow a more comprehensive description of embryo cohorts obtained from a treatment, but at extreme cost in terms of resources.

In some gene expression studies, a specific blastocyst subgroup defined on the basis of morphological characteristics may be of interest, for example expanded blastocysts when production of embryos of a quality sufficient for uterine transfer is the goal. The best culture conditions in this case are those that produce the greatest number of high-grade transferable embryos. In our experience, the comparison of stage-specific blastocysts greatly decreases the number of genes contributing to confounding effects that merely reflect the impact of the experimental treatments on embryo cohort sample heterogeneity (Plourde *et al.* 2012a, Plourde *et al.* 2012b).

How do embryos cope with stress?

One of the most comprehensive transcriptome profiling studies was a comparison of blastocysts on the basis of time spent *in vitro* or *in vivo* (Gad *et al.* 2012a). Early embryos were collected *in vivo* at different times post-insemination and their development was continued *in vitro* while embryos obtained from *in vitro* fertilization were placed *in utero* at various early developmental stages and then re-collected at the blastocyst stage for continued development. It was thus shown that extending the time *in vitro* led to affect genes implicated in lipid metabolism deviations and free radical management. These same authors have thoroughly reviewed the impact of culture conditions on transcript abundance (Gad *et al.* 2012b).

Many other large-scale gene expression studies that focused on the impact of media composition in culture systems support the conclusion that genes involved in lipid metabolism and free radical management are highly responsive cellular pathways in early embryos. For several years now, serum has been suspected of inducing abnormal offspring syndrome (Young *et al.* 2001, Lazzari *et al.* 2002) and is known to increase lipid accumulation in embryos (Crosier *et al.* 2001, McEvoy *et al.* 2001). Moreover, the stress caused by exposure to high levels of lipid affects the inflammatory response, while deviant lipid metabolism leads to decreased survival rates after cryopreservation (Rizos *et al.* 2003). Both oocytes and embryos are very sensitive to the lipid content of their microenvironment and certain fatty acids are particularly detrimental to embryo development (Leroy *et al.* 2005, Shehab-El-Deen *et al.* 2009, Leroy *et al.* 2010, Van Hoeck *et al.* 2013, Cagnone & Sirard 2014). In addition to bringing a completely new perspective to the impact of maternal nutrition on fertility, these results add emphasis to the influence of the microenvironment on embryo metabolism.

Increased concentrations of glucose cause blastomeres to shift their energetic metabolism from the standard mitochondrial aerobic pathway to the anaerobic fermentative pathway terminating with lactic acid, a sign of stress similar to the Warburg effect observed in cancer cells (Cagnone *et al.* 2012). It was shown more than a decade ago that the presence of serum not only causes lipid accumulation but also reduces mitochondrial capacity (Abe *et al.* 2002). Our studies of the impact of culture conditions based on strict comparison of the transcriptomes of morphologically staged blastocysts (early blastocysts) indicate similar effects on lipid metabolism and mitochondrial function (Plourde *et al.* 2012b). However, mitochondrial function was affected only when embryos were produced from oocytes collected from slaughterhouse ovaries. When oocytes were collected *in vivo* by trans-vaginal pick up, mitochondrial function was not affected and blastocyst rates were greatly increased (Plourde *et al.* 2012b).

It appears overall that metabolic stress in embryos is sensed primarily by mitochondria, which respond by switching energy pathways, and in turn influences cell lipid content and

free radical management, possibly triggering apoptosis. It has already been proposed that embryos of superior quality have greater mitochondrial capacity, based on higher mitochondrial DNA content (St John *et al*. 2004, El Shourbagy *et al*. 2006, Van Blerkom 2011, Stigliani *et al*. 2013). Several studies based on different methodological approaches and with different perspectives on embryo quality all converge to the conclusion that viability is highly dependent on mitochondrial status, which is highly sensitive to environmental conditions. We believe that proper mitochondrial function begins in the oocyte and translates into higher developmental potential supported by a stable and relatively quiescent metabolic state.

A window of sensitivity to stress exists around the time of embryo genome activation (Gad *et al*. 2012a). This corresponds to the time at which mitochondria switch from the immature shape found in the oocyte (known to be inefficient for oxidative phosphorylation due mainly to the limited number of cristae) to a more mature cylindrical form (Crocco *et al*. 2011). It also coincides with an increase in the efficiency of energy production from glucose (Rieger *et al*. 1992). During these important cytoplasmic events, the nuclear genome activates first by initiating transcription in the nucleolus, which is the site of ribosomal RNA production (Hyttel 2001). As mentioned, prior to genome activation, the embryo has an atypical 18S: 28S RNA ratio and subsequently acquires a ratio close to that of somatic cells (Gilbert *et al*. 2009).

Activation also involves a major genomic reprogramming that is done to erase the extremely specific programs of the gametes and allow the very plastic program of undifferentiated (totipotent or stem) cells to begin. The mechanisms by which genomic reprogramming occurs are currently the focus of many studies worldwide and are beyond the scope of the present review (for recent reviews on this topic see (Kohli & Zhang 2013, Seisenberger *et al*. 2013). This reprogramming is effected through complete reorganization of the epigenetic architecture, including DNA methylation and histone modifications, and coincides with the longest cell cycle of pre-hatching development (36.25 $\pm$ 15.05 hours in cattle (Lequarre *et al*. 2003)) and culminates in major activation of transcription.

Window of sensitivity and mechanisms of coping with stress

One of the most striking observations to emerge from comparing the transcriptome of embryos produced under different conditions is that the embryonic RNA population is composed of a large contingent of long non-coding (lnc) molecules (Robert *et al*. 2011, Zhang *et al*. 2012). Furthermore, we found that this class of transcripts is the most profoundly affected by culture conditions (Cote *et al*. 2011, Plourde *et al*. 2012b). Even lncRNA originating from the mitochondrial genome was among the candidates most affected. The *in vitro* microenvironment was also associated with the presence of lncRNA that was undetectable in blastocysts obtained *in vivo*. To our knowledge, no such variance has ever been observed for a protein-encoding transcript.

The abundance of these uncharacterized transcripts in blastomeres far exceeds the prevalence observed in somatic cells as published by the Encode Project (Harrow *et al*. 2012). The source of lncRNA has been determined from transcriptional events occurring in intergenic or intronic regions as well as from the strand opposite the protein-coding sequence, thus corresponding to natural antisense RNA (Khalil *et al*. 2009, Hawkins & Morris 2010, Li & Ramchandran 2010). These events have often been considered as transcriptional noise (Hangauer *et al*. 2013). However, emerging evidence suggests that this dark matter is important and plays a crucial role in nearly all cellular processes from nuclear transcription to epigenetic remodeling to translation control (Fatica & Bozzoni 2014). It may also be crucial for the development and maintenance of stem cell potency (van Leeuwen & Mikkers 2010, Guttman *et al*. 2011, Fatica & Bozzoni 2014).

The involvement of lncRNA in embryo physiology remains unknown. Does it result from transcriptional leakage arising from the permissive state characterizing embryonic stem cells? Stem cell transcription favors shorter gene transcripts (Mercer *et al*. 2011) due probably to a shorter cell cycle length. Is the extended cell cycle that marks the activation of the embryonic genome more prone to transcriptional errors? Are deviations in lncRNA abundance observed at the blastocyst stage indicative of denatured epigenetic marks? These questions remain unanswered.

Integration of data already available may hold the key to the future

Over the past decade, transcriptome profiling studies have been numerous. Most of these studies have focused on a handful of candidates for further characterization. The bulk of the data is still of interest insofar as each study was designed to address one or a few questions but combined with other datasets could serve to increase the statistical power to highlight trends in embryo response. Although different methodological platforms can lead to data incompatibility and increased noise, by considering the limitations regarding platform compatibility and sample composition, it may be possible to generate a reference base of embryo general responsiveness. This is important, since the concept of embryo normalcy has yet to be defined (Figure 4). For instance, to what extent does deviant management of free radicals lead to a deviant phenotype? Normalcy must reside within an interval and some stresses may be more challenging than others to embryo viability. Such a definition would provide a rational basis for improving conditions in order to produce embryos of superior quality.

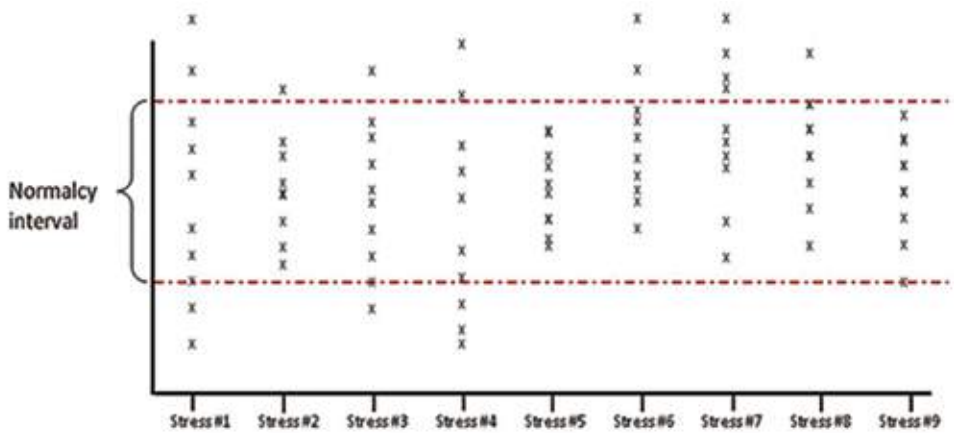

Figure 4. Given that blastocysts react to their environment, it is expected that they will modify their gene expression in response to different environmental insults represented by the different stresses along the X axis. Deviant gene expression could fall within a normalcy interval (red dots lines) that does not translate into peak phenotypes or it could induce long-term effects. The current challenge is to distinguish transient adaptive responses from deviations with potential long-term impact.

One of the main challenges ahead is to define how observed deviations in gene expression translate into physiology. Since it is now well established that embryos both react and adapt to changes in their microenvironment and that their response to the stress of assisted fertilization involves changes in gene expression level, the next step is to distinguish between transient responses and those that have a long-term impact. One way to accomplish this might be

to integrate the different layers of genomic data including transcript abundance and DNA methylation. Each of these types of information alone may be limited in usefulness while a combined picture could lead to new insight. For instance, would a deviant gene expression accompanied by altered DNA methylation at the same locus be indicative of long-term impact whereas absence of impact on DNA methylation would be more indicative of a transient response driven by transcription factors? The study of DNA methylation alone is proving to be more complex than anticipated, since methylation marks are distributed throughout the entire genome, which spans 100 times more nucleotides than the protein-coding regions. Interpretation of DNA methylation data is currently challenging to say the least. Sorting datasets in terms of extent and position of methylation marks in relation to the corresponding transcript levels may prove helpful.

Conclusion

In conclusion, transcriptome profiling of early embryos remains technically challenging, especially when the survey is conducted across developmental stages. Blastocyst gene expression profiling has provided nonetheless valuable insight into how embryos cope with their microenvironment. The possibility of adding epigenetic information will raise new opportunities to refine our knowledge of embryonic physiology. A link very likely exists between the biological functions of mRNA, lncRNA and DNA methylation. Known interactions include that involving lncRNA Xist, which drives X chromosome inactivation in females, resulting in extensive DNA methylation and shutdown of nearly all genes on this chromosome (Froberg *et al.* 2013, Lessing *et al.* 2013). Even more relevant, dysregulation in the interplay between expression of protein encoding genes, lncRNA transcription and DNA methylation is responsible for many imprinting-related disorders such as large offspring syndrome in ruminants and Beckwith-Wiedemann and Silver-Russell syndromes in humans (Chiesa *et al.* 2012, Singh *et al.* 2012, Soejima & Higashimoto 2013). With such a wealth of information within reach, the prospects for studying and defining in fine detail the characteristics of an early embryo destined to become a healthy individual are very promising.

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