Activation of the embryonic genome

M-A Sirard

Département des Sciences Animales, Centre de Recherche en Biologie de la Reproduction, Université Laval, Sainte-Foy, Québec, Canada, G1K 7P4

Activation of its genome is amongst the essential task the embryo has to undertake following fertilization of the egg. In animal and plants, this activation follows a period of transcriptional silence, which is made necessary by the requirement for an almost complete and functional reprogramming of the DNA coming from both gametes. The process by which DNA is silenced, reprogrammed and reactivated is not fully understood yet but progresses are being made, especially with the help of genomic tools. This review will focus on the recent discoveries made in different animal models and more specifically on the efforts made to further characterize the event of maternal to embryonic transition in bovine embryos.

Introduction

To simplify the reading of this review, a few definitions are required. First the differences between MET: maternal to embryonic transition MZT; maternal to zygotic transition and EGA; embryonic genome activation or ZGA; zygotic genome activation. MET refers mainly to the period during which the source of RNA being used for protein translation transitioned from maternal to embryonic. The term EGA, for "embryonic genome activation", is in fact a sub-event of the MET representing the activation of the embryonic genome required for the initiation of transcription during the MET. In MZT and ZGA, the term embryo is replaced by zygote, but because the term zygote refers to the period where both gametes fuse to form a one cell structure, the term embryo should be used as soon as the cell division occurs. The transition from maternal to embryonic RNA management is a progressive event during which maternal RNAs are depleted by translation and degradation and embryonic RNA gradually increases with the arrival of new ribosomal RNA. This review will focus on the mammalian aspect of the transition and more specifically on the information relevant to the bovine species.

MET: a common feature

The maternal to embryonic transition is not unique to the bovine, or even to animals as plants do experience such a transition between 2 generations (Baroux *et al.*, 2008). The rationale behind such re-organization is the requirement for DNA-chromatin epigenetic reprogramming, which requires a transcriptionally quiet period to ensure a proper selection of the genes to be transcribed (Baroux *et al.*, 2008).. In fact, it is likely that the epigenetic state is a major reason for the transcriptionally silent status of the embryo. This period of transcriptional arrest is often analysed using polymerase II and III inhibitors, such as alpha-amanitin, and its duration is determined by the induced developmental arrest, which varies amongst animals. This arrest can last two hours in Drosophila (Robbins (Robbins, 1980) or as much as 3 days in bovine. In mouse, it lasts one cell cycle but in species like

Corresponding author E-mail: Marc-Andre.Sirard@fsaa.ulaval.ca

Xenopus it last from 15 cell cycles for up to 3000 (Mechali *et al.*, 1990); (Schultz, 2002); (Newport & Kirschner, 1982).

MET: transcriptional silencing in animals

Three main hypotheses are being explored as the source/cause of transcriptional inhibition: 1) The chromatin is not permissive, preventing the polymerase from accessing it 2) The transcriptional machinery is either absent or not functional (ex: requiring specific post-translational modifications such as phosphorylation) and 3) Transcription is aborted by the rapid succession of cell cycles during which the absence of G_0-G_1 prevent normal transcription (Schier, 2007). These hypotheses are still in development and the evidence supporting their existence will be discussed below. To assess transcriptional activity in relation to the first hypothesis, several groups have used the sensitivity to alpha-amanitin, as an indication of requirement for further development (Mechali *et al.*, 1990). Another tool to assess transcriptional activity is the use of reporter genes In Xenopus at the pre-MBT stage (250 cells) (Newport & Kirschner, 1982) and in mouse at the male pronucleus stage (Telford *et al.*, 1990), reporter genes can be expressed and indicate that the embryos retain some capacity to process DNA information, although the structure of the chromatin associated with these vectors remains unknown.

Not only the transcription is controlled or inhibited during the early stages but the destruction of accumulated RNA is also believed to be important for the genomic activation. Recent data indicates that maternal RNA degradation is an important event for embryonic takeover. Genes like *Zelda* in drosophila (Liang *et al.*, 2008) and specific micro-RNA in trout (Ramachandra *et al.*, 2008) are believed to be specifically involved in maternal RNA degradation and indirect inducers of EGA.

MET: the chromatin status

In most mammals, the chromatin status changes through a rapid and active demethylation of the paternal genome and a more passive and progressive demethylation of the maternal genome following fertilization (Santos *et al.*, 2002). During this reprogramming process, the chromatin-induced repressive transcriptional status is believed to ensure embryonic quiescence. The repression is associated with a structural conformation preventing the association of the polymerase with the DNA. For example, the de-acetylation of H3K4 is associated with a repressive status in mouse (Bultman *et al.*, 2006). In Xenopus, the ortholog of Dnmt-1 could have a direct repressive effect on transcription independent of it's DNA methyl transferase catalytic activity that remains to be explained (Dunican *et al.*, 2008). Model animal studies indicate that embryonic genome activation is gradually established from a multi-step progression between a chromatin repressive mechanism and the establishment of the transcription machinery (Baroux *et al.*, 2008).

A recent paper from Koehler (Koehler *et al.*, 2009) illustrates how in the bovine the chromatin high order is modified at the 8 cell stage when compared to earlier stages. They have analysed microscopically the distribution of the eu vs hetero chromatin for different chromosomes (19 and 20) at the 8 cell stage, to show that they are redistributed within the nucleus, potentially to allow transcription to occur. The hypothesis is that transcription occurs mainly out of the chromosomes that are more centrally located in the nuclei.

The modification of the epigenomic marks is recognized to impact gene expression/repression as well. The epigenetic modifications can be observed on the DNA itself and the most accepted process for these type of modifications is methylation of cysteine residues which is associated with repression. Clearly, histones can also be affected by methylation, acetylation, ubiquitination, sumoylation, phospohorylation etc., and each of these modifications may have an enhancer or repressor role

depending on the site and on the level of post translational modification. For example, some histone tail sites are double or trimethylated to confer different levels of activity. In oocytes and embryos, the nucleosomes are assembled with histones that sometimes differ from "normal" somatic histones and may play inhibitory roles (McGraw *et al.*, 2006). Indeed in our laboratory, we have explored the role and abundance of bovine H1oo, oocytes-specific linker histones regulating the access of transcription factors to the DNA through nucleosome conformation, and concluded that their presence may affect transcriptional activity (McGraw *et al.*, 2006).

RNA storage

If there is a period without transcription, it almost necessarily requires the accumulation of RNA for protein formation as the proteins have a limited half-life and a cell without instructions cannot adapt to changing environment. In bovine as in other mammals and most animals, the amount of RNA that the oocytes accumulates starts to rise significantly when follicles reach their secondary phase of development (Fair *et al.*, 1997). In somatic cells, the RNA is translated or degraded within hours, therefore, the accumulation of stable in oocyte requires a complete/specific protection mechanism that has evolved in egg producing animals.

Xenopus is the most studied model to assess mRNA fate in animals. The maternal RNA is stored using a specific configuration where the mRNA is de-polyadenylated on the 3' and capped on the 5' end. The storage of mRNA is associated with ribonucleoproteins (mRNP), which represses translation. In Xenopus, there is more information about the different proteins involved in the repression of translation, one of which is *maskin*. This protein associates with the cytoplasmic polyadenylation element binding protein (CPEB) located in the 3' UTR region on mRNA that contains a cytoplasmic polyadenylation *element* (CPE). This association represses translation through the inhibitory action of maskin-elF4E located at the 5' end of the RNA (Richter, 2007). The system allows the timed translation of specific mRNA according to a combination of cytoplasmic codes acting on RNA-associated proteins interacting with the 3'UTR sequence of the stored RNAs. The activation of translation seems associated with a longer polyA tail (from 80-150 and higher) while shorted tails (around 20 A) are repressed from translation (Richter 1999). Several regulative motifs and proteins have now been identified as regulators of re-polyadenylation of maternal RNAs (Pique *et al.*, 2008). Maternal RNA must have at least 2 CPE in their 3 'UTR. These sites must be separated by 10-12 nucleotides and if there is a Pumilio-binding element (PBE) upstream of CPE, the repression is even greater. The same author proposes that the maskin protein is recruited by a CPEB dimer, which would explain the importance of the distance between the 2 CPE elements. Progressively, the 3'UTR code is being deciphered allowing our understanding of the precise spatio-temporal regulation of mRNAs during the transition from maternal to embryonic control.

Destruction of targeted maternal RNA

At least 2 degradation pathways are known to influence maternal RNA fate. The first one is controlled by maternally encoded factors and targets the 3'UTR region at specific motifs (Stitzel & Seydoux, 2007), while the second coincides with embryonic genome activation and also uses 3'UTR targets. In Drosophila, sequencing these regions has revealed families of RNA binding proteins associated with RNA fate like SMAUG and Pumilio (De Renzis *et al.*, 2007). In zebra fish, a single miRNA (miR430) drives the repression/destruction of several hundreds of maternal RNAs (Giraldez *et al.*, 2006). A similar observation with Mir-21 has been made more recently in the trout (Ramachandra *et al.*, 2008).

There is a clear demonstration of the importance of RNA degradation in Xenopus, where 2 hrs before the MBT (Mid blastula transition) a new miRNA, miR-427, appears from multimeric genes resulting in the rapid release of millions of copies in the cytoplasm (even the produced RNA is multimeric creating an average target size of 4-8kb on northern blots). The site of action of this miRNA is in the first 60 nucleotides of the 3'UTR of cyclin A1 and B2 but not related to the polyadenylation elements regulating the translation of these cyclins. Since the cyclins are involved in regulating the different phases of the cell cycle including the bypassing of the G1 phase, this help from the embryonic genome ensures the rapid and complete degradation of remaining maternal cyclins within 2 hours and a return to a more somatic cell cycle profile (Lund *et al.*, 2009).

The production of these special miRNA probably requires the activity of some polymerases and therefore requires some transcriptional capacity unless they could be stored as other RNA and release in by a post translational mechanism. Would such transcription would account for the minor genomic activation observed in a few species including the bovine (Memili & First, 1999)?

Transcriptional activation of embryonic genes

Other examples of direct and indirect actions of the embryonic genome on maternal RNA exist. In drosophila, the action of Zelda is associated with a 5'region of targeted genes activated early during embryo formation. According to microarray analyses, at least 279 genes are controlled by Zelda as indicated by their disappearance when Zelda is targeted for destruction by miR309 (Liang *et al.*, 2008). Not surprisingly, Zelda stimulates the expression of miR309, which is responsible for the rapid degradation of specific maternal RNA (Baroux *et al.*, 2008). Unfortunately, the sequence analysis of Zelda did not lead to functional orthologs in mammals. As mentioned above, specific miRNA are produced from the embryonic genome and their role is to destroy maternal RNA. In Xenopus, the transcription of these genes occurs 1-2 hours prior to the main activation of transcription and requires the RNA polymerase, as it can be inhibited by alpha-amanitin (Lund *et al.*, 2009).

If the embryonic genome takes over at the MET, does it mean that all maternal RNA is suddenly useless or destroyed? In mouse, a transcriptomic analysis of late zygotes indicates that around 60 % of de novo transcripts are novel for the embryos while the remaining 40 % was already present in the maternal pool but gets transcribed again in the embryo (Hamatani *et al.*, 2004). We can learn from the same author that a second wave of new transcripts appears between the 4-8 cell stages and the observed new gene products are likely involved in the beginning of the compaction that precedes the differentiation of ICM into trophoblast cells.

Demonstration of transcriptional silence in bovine embryos

Early experiments in the nineties demonstrated that, in the bovine, transcription is activated between the 8 and the 16 cell stage (Barnes & First, 1991). Later on, the possibility of a minor embryonic activation was suggested (Memili & First, 1999), based on the appearance of novel proteins during the 2-4 cell stage. In fact, although evidences have been provided indicating the presence of transcription before the 8 cell stage in the bovine, mainly through alpha-amanitin comparison of proteins gels (Memili & First, 2000), it has not been clearly demonstrated by experiments using specific promoters. However, promising candidates were identified by our lab during transgenic technology development experiments. We found that Beta-galactosidase reporter genes expression occurs only 60 hours post fertilization and does not depend on the number of cell divisions nor possibly fertilization as some 1 cell oocytes turned blue as an indication of transgene transcription at the same time as 8 cell embryos (Gagne *et al.*, 1995). Moreover, several authors have since used reporter genes to identify transgenic embryos using DNA vectors (Murakami *et al.*, 1999) or even

artificial chromosomes (Wang *et al.*, 2001) with the same results. Cloning can also be used to evaluate the capabilities of the transcriptional machinery (Bordignon *et al.*, 2003) but it becomes difficult to assess if the transcription observed (often as reported Green fluorescent protein expression) is due to the transfer of some cytoplasmic elements, or to the incomplete reprogramming of the transferred nuclei caused by faulty histones replacement and partial chromatin reconfiguration. Indeed, the transcriptional activity of the fused nucleus as been considered as a negative sign of reprogramming and a bad prognostic of future embryo capacity. Nevertheless, in all these cases, transgene expression is not observed before the 8-16 cell stage or a specific time after activation/fertilization (around 60 hrs). Since bacteria derived transgenes are not associated with histones but rapidly integrate resident cell histones, the chromatin status of the recipient cell is likely to be transmitted to the new DNA quite rapidly. These indirect evidences can be used to support the hypothesis that transgene transcription does not happen in early bovine embryos the same way as in somatic cells.

As mentioned above, the hypothesis of a minor transcriptional activation in bovine embryos has been described (Memili & First, 1999). As a optional explanation for the presence of new "visible RNA" in two to eight cell embryos, the possibility of maternal RNA becoming polyadenylated could be considered. This was addressed by Kanka (Kanka *et al.*, 2009) who validated candidates with specific primers during the reverse transcription to avoid the bias of polyadenylation specific priming. Therefore the question of a minor transcription period before the 8 cell stage remains to be clearly demonstrated in bovine embryos. This could be achieved by using specific promoters for new embryonic genes fused with reporter sequences and injected rapidly post fertilization. It would indicate whether transcription occurs when the right promoter is used, despite the general chromatin configuration. In this case the promoter would have specific characteristics to

Levels of RNA

One of the difficulties in assessing the transcriptional activity through RNA analysis is related to technical aspects such as extraction, reverse transcription and amplification. Because maternal RNA is sometime stored and de-polydeanylated, the length of the polyA tail is an issue. If the tail is short, it requires extraction protocols that do not use the capturing ability of poly T columns or filters. Secondly, if the primers used for the reverse transcription include a polyT sequence, it will exclude an undefined portion of the stored RNA and finally, if a T7 polymerase is used for RNA amplification (as is often the case for microarray experiments), the presence of a polyA tail will likely make a difference since non polyadenylated RNA may not be necessarily amplified. Therefore, much more care should be given to the interpretation of RNA levels prior to the 8 cell stage. These technological considerations have been clearly demonstrated in several papers from our laboratory (Gilbert *et al.*, 2009a; (Gilbert *et al.*, 2009b). In fact, we have seen several examples where the amount of RNA measured with Q-PCR inversely correlated with the protein levels measured by western blots (Vigneault *et al.*, 2009). Therefore, the use of terms like "up regulation" or "down regulation" should be used only from the 8 cell stage onward and in comparison with the proper alpha amanitin controls before the blastocyst stage (Vigneault *et al.*, 2009).

Microarrays analysis of embryonic genome

Although potentially very rich in information as they can probe thousands of gene to assess their level of expression, microarrays data are to be used with caution especially with early embryos. In a recent review by Evsikov (Evsikov & Marin de Evsikova, 2009), several problems are described that users should consider before doing or interpreting microarray data. Firstly, the targets are limited in length and therefore only represent a small portion of the gene they are supposed to indicate. The fact that only 39 % of the changes observed on microarrays are

associated with variation of a precise target gene is also somewhat frightening (Kwan *et al.*, 2008). The other 61 % represent initiation and termination sites variants or spliced variants. Secondly, microarrays are not suitable for quantification assays since, in the case of oocytes and embryos, the material needs to be amplified and such amplification creates numerous bias (Gilbert *et al.*, 2009a; Gilbert *et al.*, 2009b). Even in mouse, a 2-cell embryo contains 0.26 pg of RNA, which is four times less than oocytes at 0.95 pg of mRNA (Evsikov & Marin de Evsikova, 2009). The normal methodology promotes the use of equal amounts of mRNA for different biological samples, which is misleading when comparing a comparable number of embryos from different stages unless one can measure very accurately the total amount of each sample and use the exact same. Indeed when we compared ten 8-cells with 10 GV after the same amplification process we have at the end the same amount of aRNA although initially the GV contains several times more RNA than the 8 cells meaning that the genes present in the latter will be over amplified since the process is not linear up to the end, otherwise the end result would be different. Thirdly, due to cross hybridization within gene families, there are several instances when it becomes impossible to assess a specific gene product to a target as the target may hybridize with several genes products from the same family (Kwan *et al.*, 2008). Another problem is the fact that several amplification protocols use the T-7oligo(dT) primer in their first step, indirectly selecting mRNA that have a poly A tail and potentially excluding several of the stored mRNA that are de-polyadenylated, as stated above. Our laboratory has demonstrated such a bias using different primers and real time PCR with pre-MET embryos (Vigneault *et al.*, 2007).

Genes "associated" with the genome activation

It is interesting to look at the functions of genes that are either targeted for destruction or protected from it during the first wave of transcription of the embryonic genome. In drosophila, Gene ontology identified that candidate genes that are destroyed mainly belong to the family of cell cycle proteins, while the conserved ones belong to the transcription factors or RNA transaction processes (binding metabolism, translation) families (Tadros & Lipshitz, 2009). In mouse, unstable mRNA (the ones that disappear) are also associated with the cell cycle but the RNA associated with RNA processing seems to come from the new genome rather than from the protected maternal pool (Hamatani *et al.*, 2004). The mouse maternal to embryonic transition is difficult to analyse, principally due to the rapid activation of the embryonic genome at the end of the first cell cycle when the first division occurs. The microarray information is only partially analysed and it will take further investigations and demonstration to identify the important factors (Li *et al.*; Evsikov & Marin de Evsikova, 2009). Nevertheless, it is clear from the mouse studies that a number of specific factors or transcripts eventually translated into proteins are required for the proper genomic function of the embryo. Factors like Dicer, Ago2 (eukaryotic elongation factor 2c), Atg5 (autophagy related 5) are required to degrade maternal factors (Tsukamoto *et al* 2008). Gene products like *Hr6a* (*Ubiquitin conjugating enzyme E2A*), *Nucleoplasmin 2*, *Tif1a* (*tripartite motif containing 24*) and *Smarca4* are required for chromatin remodelling. Important transcription factor as Oct-4 and Sox 2 influence competence as well as differentiation of blastomeres (Zuccotti *et al.*, 2009). The re-establishment of methylation with Dnmt1 (which also act on chromatin accessibility) and Dnmt3a, involved in de novo methylation, and their impact on the ability of DNA to be transcribed, are to be linked with specific gene activation/inactivation. To this group of better known actors, some new or oocytes specific genes are slowly being added as they are being characterized: Zar-1, *Mater*, Padi6 and Filia as upstream actor of oocyte differentiation (Li *et al.*). The regulation of

developmental competence may include the interplay between Oct-4, *Stella* and GCNF (germ cell nuclear factor) during oocyte growth and should be crucial at the end of oocytes maturation (Zuccotti *et al.*, 2009).

Using the rabbit, in which embryonic activation occurs later than the mouse, and is more similar to bovine and human, Léandri made subtractive libraries from early morula and 4 cells embryos (Leandri *et al.*, 2009). The authors used cluster analysis to organize the genes according to their rise or demise from the 4 cell stage to blastocyst. Some of the clustered categories associated with a rise after 4 cells (putatively new embryonic messages, validated by Q-PCR) contain candidates that are common with the bovine. In bovine, the subtraction libraries obtained between the 8 cell plus or minus alpha-amanitin is, like the rabbit, very much enriched with RNA processing elements (Vigneault *et al.*, 2009). Although the list of embryonic early genes is not very long (< 200 genes) several were found in common; *H2Afz* (*histone H 2A*), *SAP18* (*Sin-3 associated proteins*), *SLC family* (*solute carrier family*), *Mthfd1* (*Methylenetetrahydrofolate deshydrogenase*), *ATP 5 b* (*ATP synthase*), *EIF5* (*eukaryotic translation initiation factor 5*) albeit several EIF members are present in both groups, they do not necessarily have the same subtype. Considering that these subtracted libraries are necessarily incomplete and even very partial, the number of similarities is interesting and reassuring for the comparative biology point of view. Not surprisingly, several maternal genes that do decrease during the MET are also common between the rabbit and the bovine studies (Leandri *et al.*, 2009) (Vigneault *et al.*, 2009).

In the pig, embryonic genome activation also occurs later than in the mouse and closer to the rabbit at the 4 cell stage. A recent review from Prather indicates the usefulness of the genomic tools (microarrays and subtractive libraries) to identify the players involved in this process (Prather *et al.*, 2009). In a previous study, they used an array dedicated to this species, even though the genome is incomplete, to profile the transcriptome of the MET period in pig embryos (Whitworth *et al.*, 2005). They have uncovered the importance of several processes such as macroautophagy, which is important for the removal of maternal proteins. This is also being observed in our study in bovine (see below). In the pig, the role of micro RNA also seems important for the degradation of maternal material as loss of function of *EIF2C1* results in the stabilization of specific maternal RNA and development arrest during the MET. *EIF2C1* is part of the RNA-Induced Silencing Complex (RISC) required to process miRNA (Whitworth *et al.*, 2005). However, it is important to note that the role of Dicer and RISC components specifically at the MET are not well understood. The long term effects of disrupting these components are not just important but critical to survival.

Bovine microarrays results

Several recent papers have been published about the MET in bovine and the objective here is not to repeat what has been published but to briefly summarize the pre-and post genomic analysis and then to analyse new results from specific experiments that uncovered genes involved in the bovine MET.

A recent paper from Bettegowda (Bettegowda *et al.*, 2008) has made a list of important genes that are inherited from the maternal pool for continued development or proper embryonic activation. When disrupted, genes like *mater* (Pennetier *et al.*, 2006) *Zar-1*(Pennetier *et al.*, 2004) *NPM2* (Vallee *et al.*, 2008) lead to embryonic arrest phenotypes in the mouse and are present in bovine, but others like *stella*, *HsF1* (*heat shock factor 1*) *RAD 6* and *basonuclin* have not been identified yet in bovine (Bettegowda *et al.*, 2008). The same group has recently uncovered the importance of a new gene; *importin-alpha 8* or *KPNA7* as a nuclear importer

of specific cargo like DNMT1 and HDAC4 and potentially involved in embryonic initiation of transcription (Tejomurtula *et al.*, 2009). Another paper in 2009 (Thelie *et al.*, 2009) confirmed the absence of transcription during the oocyte maturation period and that most (92%) of the maternal transcripts present in oocytes do not re-appear in the blastocyst.

In our laboratory, we have generated a subtractive library to probe the bovine transcriptome in the 8 cell embryo and compare it to 8 cells treated with alpha-amanitin (Vigneault *et al.*, 2009). We found clear evidences that some biological processes are prioritized in the early 8 cell. When the publication of Vigneault et al was submitted 2 years ago, we had limited tools to analyse the system biology value of the data generated. Nonetheless, the lists themselves as organized by functions or GO terms are truly pointing in very specific directions:

- Transcription regulation: 37 transcripts with 50% higher than 5 folds differences
- RNA processing: 40 transcripts with 33% higher than 5 fold differences
- Protein biosynthesis: 38 transcripts with 50 % higher than 5 fold differences
- DNA replication/ nucl. biosynthesis:15 transcripts with 33 % higher than 5 fold
- Protein degradation/modification: 25 transcripts with 50% higher than 5 fold

These 5 GO terms divisions represent the large majority of the known functions associated with the transcript of embryonic origin.

This original bovine data set was then introduced into IPA (Ingenuity pathway analysis). This program uses orthologs for human-mouse or rat to assess links between genes based on the available literature. This program is quite powerful but requires some efforts to ensure a proper analysis with embryonic tissues. Most of the published annotations are associated with the 3 mammalian species above, but since a large portion of transcriptomic studies are derived from cancer analyses, the dataset is enriched with ontogenic relations. Nevertheless, it is quite a powerful tool compared to individual gene search. When the transcriptome is analysed with bovine 8 cells compared to 8 cells with alpha-amanitin, all the differences is on one sided, or "up regulated", in the control without polymerase inhibitor, which makes a lot of sense. Therefore, on the network provided by IPA, all the genes coming from the bovine comparison will be displayed from pink to red depending on the relative intensity of the increased amount of RNA measured. The analysis revealed 11 networks of 15 related molecules or more (table 1). The score indicate an arbitrary value for the Ingenuity analysis which is dependant of the number of molecules involved in a given network (focus molecules)

The functions described in table 1 are in agreement with what is believed to happen at that stage: the resumption of transcription. The visual description of the network is also quite informative. Some example will be given here to highlight particular pathways of interest. The first network is illustrated in figure 1. The first node of interest involves *PSMA*, *PSMA 2*, *PSMA3*, *PSMA6*, *20 S proteasome*, *proteasome 700*, *immunoproteasome pa28*, *26 S proteasome* and *prion protein*. This group fits with the requirement of maternal protein degradation associated with MET in several species (see above). To observe nine interrelated transcripts that are all "upregulated" (see previous comments about the term upregulation) concomitantly is a strong indication that this is not a minor event but an important action in the embryonic program. In addition, the presence of *Snrp*, the transcription elongation factors (TCE family) and the eukaryotic translation elongation factors all indicate the activation of transcription (SKP1 is a kinase involved in transcription regulation), which is even more obvious in network 2.

Table 1. IPA function analysis of the 11 major network at MET in Bovine embryos.

ID	Score	Focus molecules	Top Functions
1	49	28	RNA Post-Transcriptional Modification, Molecular Transport, Nucleic Acid Metabolism
2	42	25	Protein Synthesis, RNA Post-Transcriptional Modification, DNA Replication, Recombination, and Repair
3	41	25	Cancer, Gene Expression, Cell Cycle
4	31	20	RNA Post-Transcriptional Modification, Protein Synthesis, Cellular Assembly and Organization
5	27	19	Protein Synthesis, Cell Death, Gene Expression
6	25	17	Molecular Transport, RNA Trafficking, RNA Post-Transcriptional Modification
7	23	16	Cell Cycle, Connective Tissue Development and Function, Cancer
8	21	16	Organismal Injury and Abnormalities, Neurological Disease, Behavior
9	21	15	Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking
10	19	14	Cell Signaling, Cellular Assembly and Organization, Cellular Function and Maintenance
11	19	14	Cell Cycle, Cellular Assembly and Organization, Cell Death

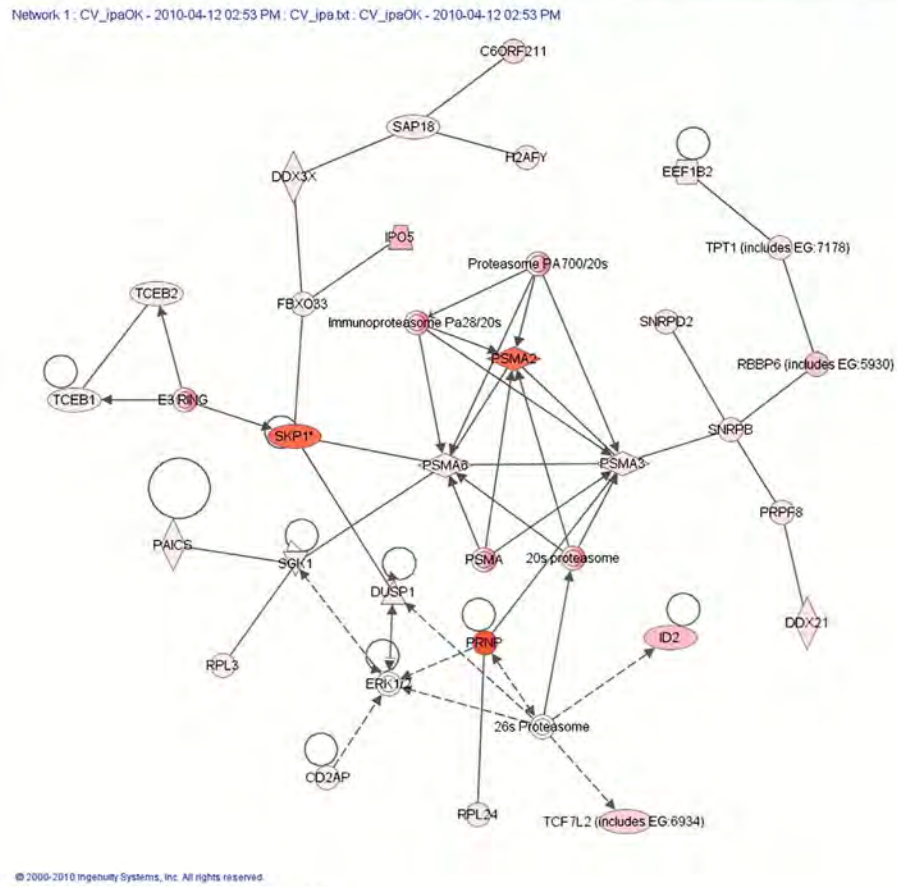

Fig. 1. Illustration of Network 1.

The second network (figure 2) focuses even more on transcriptional activation as several elongation factors are linked together. The concomitant presence of 3 *HNRNP, A2B1* and *A1*, 5 eukaryotic elongation factors *EIF 5, 3A, 3H, 4g, 4G2* and 2 types of *histones H3* associated with active transcription and some importins and transcription regulators such as *ATF1* and *GABPA*, splicing factors as *SF3, B1* and *B3*, indicates that transcriptional activation occurs in the nucleus. The IPA system adds some complementary information to fill the networks and these are not coloured (*Pka, ERK 1-2, Cyclin A*) but indicate potential regulatory mechanisms to investigate.

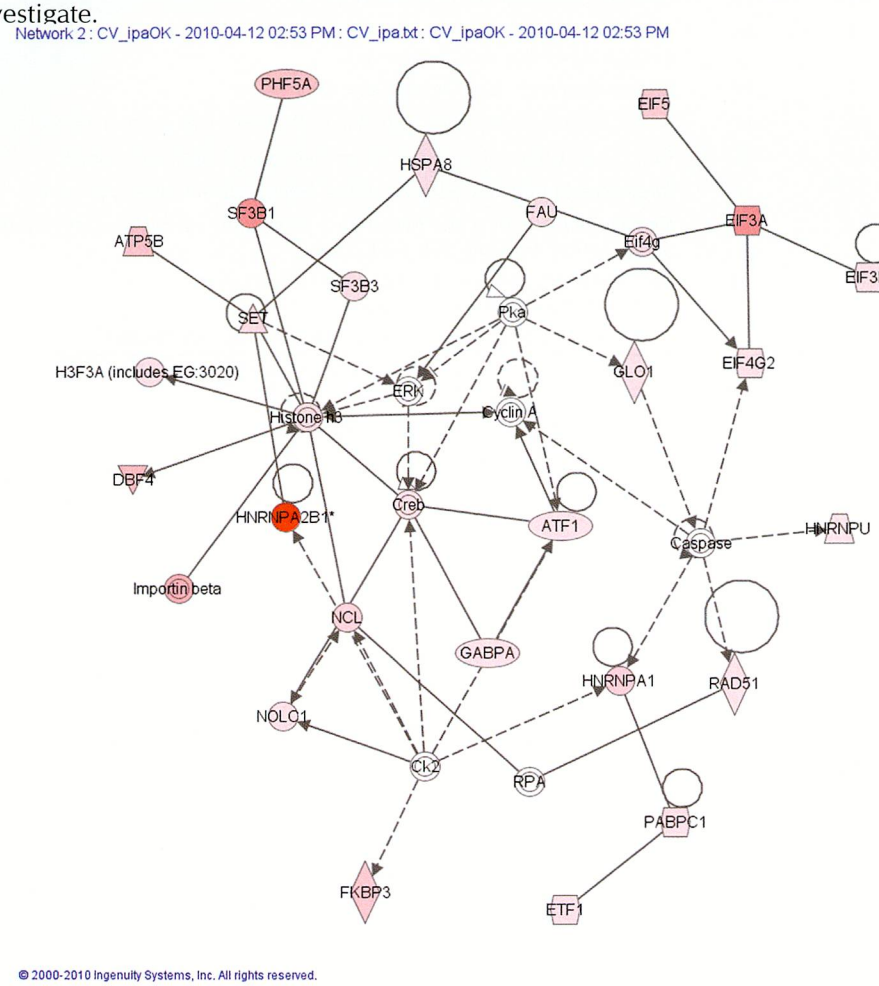

Fig. 2. Illustration of Network 2.

Network 3 (figure 3) also indicates a rise in transcriptional activity as it contains 2 *TBP* (*tata box bonding proteins*), *TAF 7* and *9*, 2 *KLF* genes, *KAT2B*, *NFYA*, *YY1* and *REST,* which are all transcriptional regulators. It is interesting to note that the estrogen receptor is linked with 3 of these transcription regulators (*KAT2B*, *TAF9* and *KLF10*).

The last network to be presented, network 4 (figure 4), is interesting by the finding of a potentially important node in a growth network : *GRB2, growth factor receptor bound protein 2*, which has several molecular functions such as phosphotyrosine binding, SH3/SH2 adaptor activity, epidermal growth factor receptor binding, protein domain specific binding, insulin receptor substrate binding and is involved in the following cellular processes: the MAPKKK

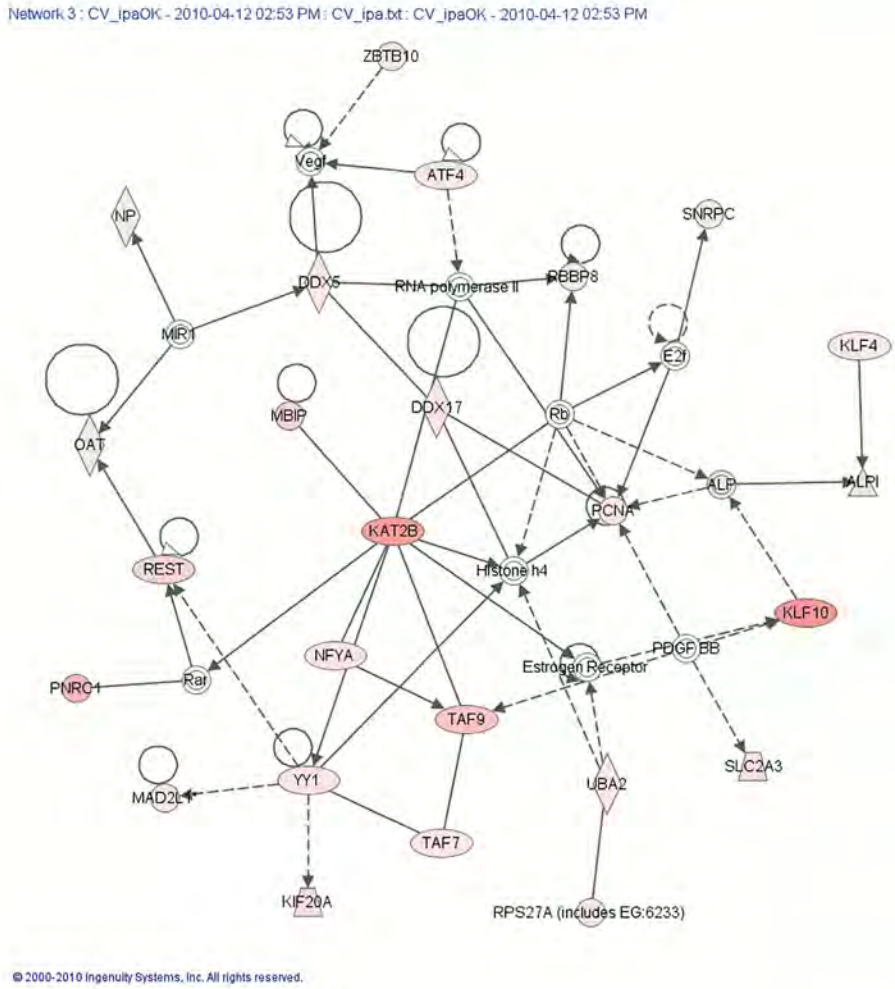

Fig. 3. Illustration of Network 3.

cascade, the epidermal growth factor receptor signalling pathway, Ras protein signal transduction, cell-cell signalling, aging, the insulin receptor signalling pathway, cell differentiation, interspecies interaction between organisms and protein hetero-oligomerization. As it can be observed in figure 4, *GRB2* acts on transcription factors (*MATR3*), on 3 types of ribonucleoproteins (*HNRNP* types *R*, *PF* and *H1*), and on 13 different ribosomal proteins *2,7, 10 A 18, 24, 26, 27, 35 , 37 A, S14, S16, S18* and *S23*. As this *GRB2* was not present in the limited library that was used, it would indicate that we missed it before the use of IPA.

Conclusion

The image behind the puzzle is slowly forming. Looking at the data from other species, particularly the mouse, where single genes can be analyzed using the Knock out approach, the mechanisms of embryonic activation are progressively being revealed. It seems that transcriptional arrest is almost universal in animals although the relationship between the epigenome and transcriptional arrest is still actively investigated and debated. The maternal RNA and

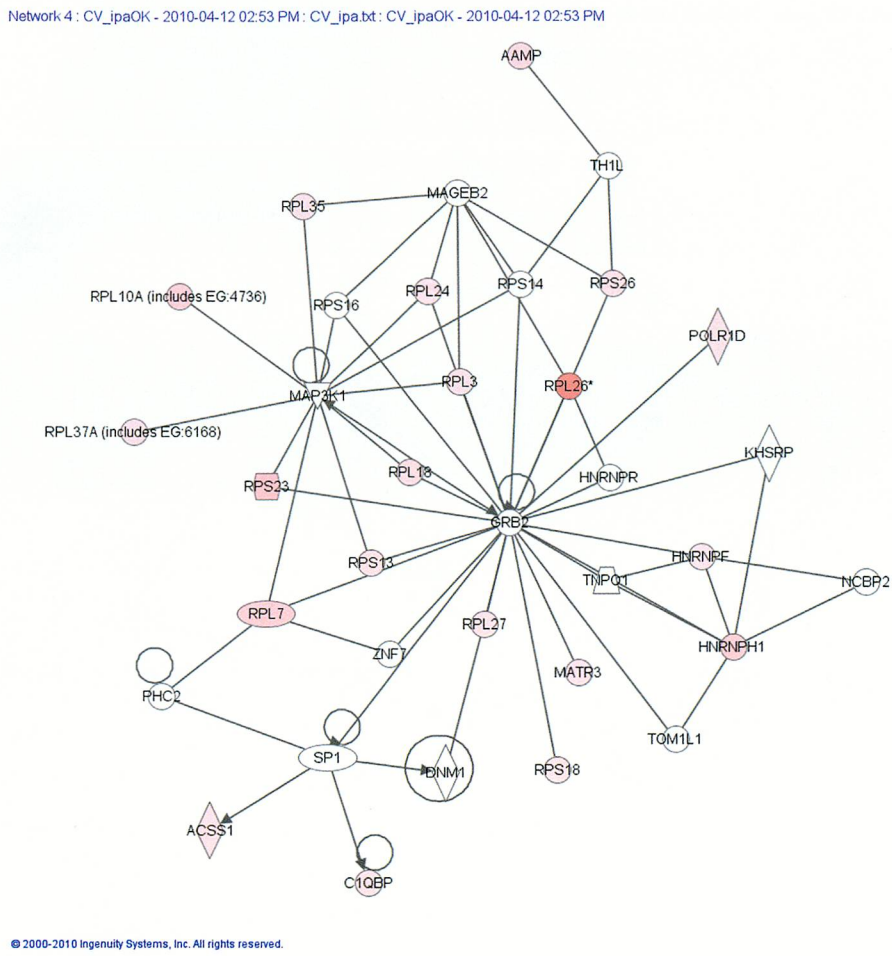

Fig. 4. Illustration of Network 4.

oocyte's proteins are important to trigger embryonic activation as specific products are required for this process, but at the same time the stock pile must be dismantled or destroyed to ensure proper function or activation of the new embryonic genome. The role of miRNAs is confirmed in many vertebrate and invertebrates models but is not defined clearly yet in mammals, albeit the removal of Dicer or other RISC components results in developmental consequences, suggesting an important function of this regulatory mechanism. However, it is important to note that the role of Dicer and RISC components specifically at the MET are not well understood. The long term effects of disrupting these components are not just important but critical to survival. The picture in bovine is getting in line with what has been observed in other mammals and indicates a multistep transcriptional activation around the 8 cell stage. Our findings further suggest that crucial functions are being activated in the early 8 cells, such as protein degradation, ribosome formation and the production of several transcriptional factors and units required for gene expression. These functional analyses are promoting the formulation of new hypotheses for the regulation of this process as new nodes and pathways are being exposed for further testing.

Acknowledgements

The author would like to thank Christian Vigneault for producing the bovine libraries and hybridization results and Julie Nieminen for manuscript revision. Financial support was obtained from NSERC (Natural Sciences and Engineering Research Council of Canada).

References

Barnes F L and First N L. 1991. Embryonic transcription in in vitro cultured bovine embryos. *Mol Reprod Dev*, **29**:117-123.

Baroux C, Autran D, Gillmor C S, Grimanelli D and Grossniklaus U. 2008. The maternal to zygotic transition in animals and plants. *Cold Spring Harb Symp Quant Biol*, **73**:89-100.

Bettegowda A, Lee K B and Smith G W. 2008. Cytoplasmic and nuclear determinants of the maternal-to-embryonic transition. *Reprod Fertil Dev*, **20**:45-53.

Bordignon V, Keyston R, Lazaris A, Bilodeau A S, Pontes J H, Arnold D, Fecteau G, Keefer C and Smith L C. 2003. Transgene expression of green fluorescent protein and germ line transmission in cloned calves derived from in vitro-transfected somatic cells. *Biol Reprod*, **68**:2013-2023.

Bultman S J, Gebuhr T C, Pan H, Svoboda P, Schultz R M and Magnuson T. 2006. Maternal BRG1 regulates zygotic genome activation in the mouse. *Genes Dev*, **20**:1744-1754.

De Renzis S, Elemento O, Tavazoie S and Wieschaus E F. 2007. Unmasking activation of the zygotic genome using chromosomal deletions in the Drosophila embryo. *PLoS Biol*, **5**:e117.

Dunican D S, Ruzov A, Hackett J A and Meehan R R. 2008. xDnmt1 regulates transcriptional silencing in pre-MBT Xenopus embryos independently of its catalytic function. *Development*, **135**:1295-1302.

Evsikov A V and Marin de Evsikova C. 2009. Gene expression during the oocyte-to-embryo transition in mammals. *Mol Reprod Dev*, **76**:805-818.

Fair T, Hulshof S C, Hyttel P, Greve T and Boland M. 1997. Nucleus ultrastructure and transcriptional activity of bovine oocytes in preantral and early antral follicles. *Mol Reprod Dev*, **46**:208-215.

Gagne M, Pothier F and Sirard M A. 1995. Effect of microinjection time during postfertilization S-phase on bovine embryonic development. *Mol Reprod Dev*, **41**:184-194.

Gilbert I, Scantland S, Dufort I, Gordynska O, Labbe A, Sirard M A and Robert C. 2009a. Real-time monitoring of aRNA production during T7 amplification to prevent the loss of sample representation during microarray hybridization sample preparation. *Nucleic Acids Res*, **37**:e65.

Gilbert I, Scantland S, Sylvestre E L, Gravel C, Laflamme I, Sirard M A and Robert C. 2009b. The dynamics of gene products fluctuation during bovine pre-hatching development. *Mol Reprod Dev*, **76**:762-772.

Giraldez A J, Mishima Y, Rihel J, Grocock R J, Van Dongen S, Inoue K, Enright A J and Schier A F. 2006. Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science*, **312**:75-79.

Hamatani T, Carter M G, Sharov A A and Ko M S. 2004. Dynamics of global gene expression changes during mouse preimplantation development. *Dev Cell*, **6**:117-131.

Kanka J, Kepkova K and Nemcova L. 2009. Gene expression during minor genome activation in preimplantation bovine development. *Theriogenology*, **72**:572-583.

Koehler D, Zakhartchenko V, Froenicke L, Stone G, Stanyon R, Wolf E, Cremer T and Brero A. 2009. Changes of higher order chromatin arrangements during major genome activation in bovine preimplantation embryos. *Exp Cell Res*, **315**:2053-2063.

Kwan T, Benovoy D, Dias C, Gurd S, Provencher C, Beaulieu P, Hudson T J, Sladek R and Majewski J. 2008. Genome-wide analysis of transcript isoform variation in humans. *Nat Genet*, **40**:225-231.

Leandri R D, Archilla C, Bui L C, Peynot N, Liu Z, Cabau C, Chastellier A, Renard J P and Duranthon V. 2009. Revealing the dynamics of gene expression during embryonic genome activation and first differentiation in the rabbit embryo with a dedicated array screening. *Physiol Genomics*, **36**:98-113.

Li L, Zheng P and Dean J. 2010.Maternal control of early mouse development. *Development*, **137**:859-870.

Liang H L, Nien C Y, Liu H Y, Metzstein M M, Kirov N and Rushlow C. 2008. The zinc-finger protein Zelda is a key activator of the early zygotic genome in Drosophila. *Nature*, **456**:400-403.

Lund E, Liu M, Hartley R S, Sheets M D and Dahlberg J E. 2009. Deadenylation of maternal mRNAs mediated by miR-427 in Xenopus laevis embryos. *RNA*, **15**:2351-2363.

McGraw S, Vigneault C, Tremblay K and Sirard M A. 2006. Characterization of linker histone H1FOO during bovine in vitro embryo development. *Mol Reprod Dev*, **73**:692-699.

Mechali M, Almouzni G, Andeol Y, Moreau J, Vriz S, Leibovici M, Hourdry J, Geraudie J, Soussi T and Gusse M. 1990. Genes and mechanisms involved in early embryonic development in Xenopus laevis. *Int J Dev Biol*, **34**:51-59.

Memili E and First N L. 1999. Control of gene expression at the onset of bovine embryonic development. *Biol Reprod*, **61**:1198-1207.

Memili E and First N L. 2000. Zygotic and embryonic gene expression in cow: a review of timing and mechanisms

of early gene expression as compared with other species. *Zygote*, **8**:87-96.

Murakami M, Fahrudin M, Varisanga M D and Suzuki T. 1999. Fluorescence expression by bovine embryos after pronuclear microinjection with the EGFP gene. *J Vet Med Sci*, **61**:843-847.

Newport J and Kirschner M. 1982. A major developmental transition in early Xenopus embryos: I. characterization and timing of cellular changes at the midblastula stage. *Cell*, **30**:675-686.

Newport J and Kirschner M. 1982. A major developmental transition in early Xenopus embryos: II. Control of the onset of transcription. *Cell*, **30**:687-696.

Pennetier S, Perreau C, Uzbekova S, Thelie A, Delaleu B, Mermillod P and Dalbies-Tran R. 2006. MATER protein expression and intracellular localization throughout folliculogenesis and preimplantation embryo development in the bovine. *BMC Dev Biol*, **6**:26.

Pennetier S, Uzbekova S, Perreau C, Papillier P, Mermillod P and Dalbies-Tran R. 2004. Spatio-temporal expression of the germ cell marker genes MATER, ZAR1, GDF9, BMP15,andVASA in adult bovine tissues, oocytes, and preimplantation embryos. *Biol Reprod*, **71**:1359-1366.

Pique M, Lopez J M, Foissac S, Guigo R and Mendez R. 2008. A combinatorial code for CPE-mediated translational control. *Cell*, **132**:434-448.

Prather R S, Ross J W, Isom S C and Green J A. 2009. Transcriptional, post-transcriptional and epigenetic control of porcine oocyte maturation and embryogenesis. *Soc Reprod Fertil Suppl*, **66**:165-176.

Ramachandra R K, Salem M, Gahr S, Rexroad C E, 3rd and Yao J. 2008. Cloning and characterization of microRNAs from rainbow trout (Oncorhynchus mykiss): their expression during early embryonic development. *BMC Dev Biol*, **8**:41.

Richter J D. 2007. CPEB: a life in translation. *Trends Biochem Sci*, **32**:279-285.

Robbins L G. 1980. Maternal-zygotic lethal interactions in Drosophila melanogaster: the effects of deficiencies in the zeste-white region of the X chromosome. *Genetics*, **96**:187-200.

Santos F, Hendrich B, Reik W and Dean W. 2002. Dynamic reprogramming of DNA methylation in the early mouse embryo. *Dev Biol*, **241**:172-182.

Schier A F. 2007. The maternal-zygotic transition: death and birth of RNAs. *Science*, **316**:406-407.

Schultz R M. 2002. The molecular foundations of the maternal to zygotic transition in the preimplantation embryo. *Hum Reprod Update*, **8**:323-331.

Stitzel M L and Seydoux G. 2007. Regulation of the oocyte-to-zygote transition. *Science*, **316**:407-408.

Tadros W and Lipshitz H D. 2009. The maternal-to-zygotic transition: a play in two acts. *Development*, **136**:3033-3042.

Tejomurtula J, Lee K B, Tripurani S K, Smith G W and Yao J. 2009. Role of importin alpha8, a new member of the importin alpha family of nuclear transport proteins, in early embryonic development in cattle. *Biol Reprod*, **81**:333-342

Telford N A, Watson A J and Schultz G A. 1990. Transition from maternal to embryonic control in early mammalian development: a comparison of several species. *Mol Reprod Dev*, **26**:90-100.

Thelie A, Papillier P, Perreau C, Uzbekova S, Hennequet-Antier C and Dalbies-Tran R. 2009. Regulation of bovine oocyte-specific transcripts during in vitro oocyte maturation and after maternal-embryonic transition analyzed using a transcriptomic approach. *Mol Reprod Dev*, **76**:773-782.

Tsukamoto S, Kuma A, Murakami M, Kishi C, Yamamoto A, Mizushima N. 2008. Autophagy is essential for preimplantation development of mouse embryos. Science Jul4;321(5885):117-20

Vallee M, Aiba K, Piao Y, Palin M F, Ko M S and Sirard M A. 2008. Comparative analysis of oocyte transcript profiles reveals a high degree of conservation among species. *Reproduction*, **135**:439-448.

Vigneault C, Gilbert I, Sirard M A and Robert C. 2007. Using the histone H2a transcript as an endogenous standard to study relative transcript abundance during bovine early development. *Mol Reprod Dev*, **74**:703-715.

Vigneault C, Gravel C, Vallee M, McGraw S and Sirard M A. 2009. Unveiling the bovine embryo transcriptome during the maternal-to-embryonic transition. *Reproduction*, **137**:245-257.

Vigneault C, McGraw S and Sirard M A. 2009. Spatio-temporal expression of transcriptional regulators in concert with the maternal-to-embryonic transition during bovine in vitro embryogenesis. *Reproduction*, **137**:13-21.

Wang B, Lazaris A, Lindenbaum M, Stewart S, Co D, Perez C, Drayer J and Karatzas C N. 2001. Expression of a reporter gene after microinjection of mammalian artificial chromosomes into pronuclei of bovine zygotes. *Mol Reprod Dev*, **60**:433-438.

Whitworth K M, Agca C, Kim J G, Patel R V, Springer G K, Bivens N J, Forrester L J, Mathialagan N, Green J A and Prather R S. 2005. Transcriptional profiling of pig embryogenesis by using a 15-K member unigene set specific for pig reproductive tissues and embryos. *Biol Reprod*, **72**:1437-1451.

Zuccotti M, Merico V, Redi C A, Bellazzi R, Adjaye J and Garagna S. 2009. Role of Oct-4 during acquisition of developmental competence in mouse oocyte. *Reprod Biomed Online*, **19 Suppl 3**:57-62.

Zuccotti M, Merico V, Sacchi L, Bellone M, Brink T C, Stefanelli M, Redi C A, Bellazzi R, Adjaye J and Garagna S. 2009. Oct-4 regulates the expression of Stella and Foxj2 at the Nanog locus: implications for the developmental competence of mouse oocytes. *Hum Reprod*, **24**:2225-2237.