

The use of genomics and proteomics to understand oocyte and early embryo functions in farm animals

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Oocyte maturation, a simple and visible phenomenon, is about to be transformed into a complex and not so visible molecular cascade leading to the marking of the following generation. The study of oocyte maturation in mammals is progressively changing towards a more molecular approach. This review addresses the main challenges in the study of RNA extraction and quantification in oocytes and embryos as well as the importance of the mRNA maturation. The identification of specific genes in oocytes and embryos is now possible with the use of powerful tools, such as library analysis or subtractions, DNA array, comparative analysis of databanks from other mammals or animals and two-dimensional gel electrophoresis analysis. Finally, RNA interference is a useful tool for studying gene function by knocking out the activity of specific genes and will be used in oocytes and embryos.

Oocyte maturation

Culture of immature oocytes of many ruminant species has resulted in thousands of live births in species such as cows. However, the physiological events that occur during oocyte maturation are still poorly understood. What we know is based mainly on what we see. Powerful microscopy can be used to assess the morphology, ultrastructure and modifications of the organelles over time (Hyttel *et al.*, 1997). These observations are the basis of what is known as nuclear maturation, in which the nucleus of the oocyte moves from the germinal vesicle (GV) to the metaphase II stage when it stops until sperm entry to complete the meiotic process. Microscopy can also reveal ultrastructural modifications to the organelles of the oocyte, for example the migration of cortical granules, the re-positioning of mitochondria and the cytoplasmic contraction from the zona pellucida. Collectively, these changes are known as cytoplasmic maturation. Cytoplasmic maturation is also required for a normal interaction with spermatozoa but a number of components of these physiological changes are not visible even under the most powerful microscope. It is now clear that the most important part of oocyte maturation is molecular maturation. Although often confounded with cytoplasmic maturation, molecular maturation is becoming an important subset of cytoplasmic maturation in our understanding of the physiology of the ovary, oocyte and embryo (Fig. 1).

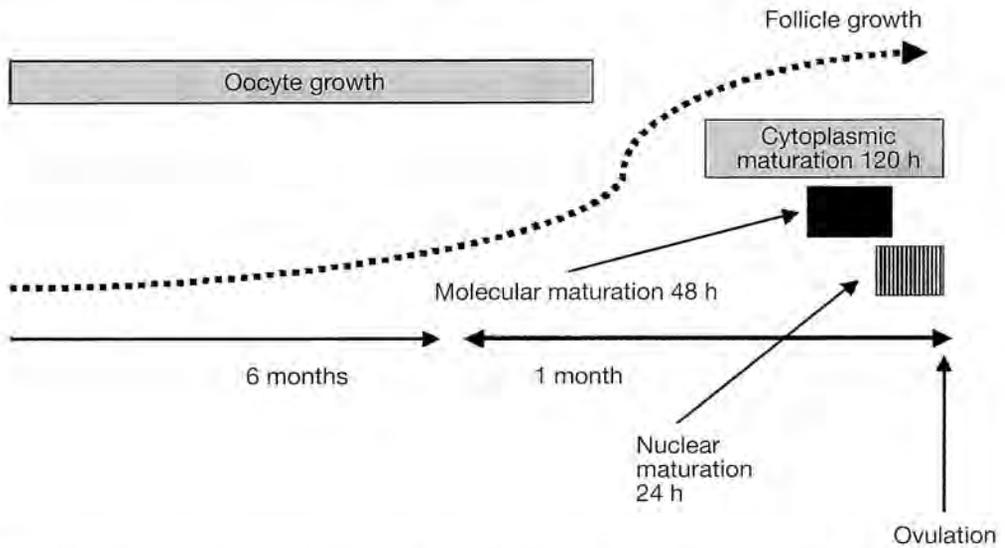


Fig. 1. Representation of the time-line that locates the molecular, nuclear and cytoplasmic maturation of the bovine oocyte. Cytoplasmic maturation: required to interact with spermatozoa. Molecular maturation: required to develop to blastocyst. Nuclear maturation: required for normal diploidy.

Recently, it has been shown in ruminants that the capacity of the oocyte to become an embryo could be pre-programmed in the ovary. Indeed, when suitable hormonal treatment is administered to a cow before follicular aspiration, it is possible to harvest immature oocytes that will almost all become embryos (Blondin *et al.*, 2002). This hormonal treatment simply mimics the natural ovulatory process, whereby FSH increases and subsequently decreases a few days before ovulation. The difference is that progesterone does not decrease to allow ovulation and the follicles start their progression towards atresia. However, the appropriate signals have been given to the oocyte and when removed from the compromised follicle, the oocyte can express its competence *in vitro*. This situation is slightly different from that in the mouse in which fully grown oocytes have already acquired the ability to develop into embryos. A similar approach applied to polycystic ovary syndrome in humans indicates that this delayed competence may be conserved among large mammals (Trounson *et al.*, 1994; Mikkelsen and Lindenberg, 2001).

At present, it is known that a given treatment to the animal induces competence in bovine oocytes, but how do we find the cause or the mechanism? Except for slight changes in the morphology of the nucleolus that is associated with the prematuration or oocyte capacitation event (Hyttel *et al.*, 1997), there are no methods to distinguish between competent and incompetent oocytes other than IVF culture and embryo transfer. However, the difference is present in the form of molecular memory. We know that what happens during the last few hours before oocyte collection will have a significant impact on further competence. This event must be memorized or stored in molecules with a long half-life. In most tissues, long-term effects are mediated by gene expression, but the oocyte rapidly loses its transcriptional capacity upon maturation. The second most preferred mechanism to retain information is in proteins and possibly competent oocytes accumulate a number of specific and important factors for later use in embryogenesis (Rodman and Bachvarova, 1976). However, studies in vertebrates, such as *Xenopus* (McGrew *et al.*, 1989), mice (Huarte *et al.*, 1992) and in insects

(*Drosophila*) (Lieberfarb *et al.*, 1996) indicate that stored mRNA might be the preferred form of information storage in oocytes. The problem with mRNA is related to its instability or half-life. In most somatic cells, the half-life of mRNA is from minutes to hours, whereas in oocytes mRNA can remain stable for days (Brower *et al.*, 1981).

In vertebrates, a number of unique mechanisms have been found to mask the 3' and 5' ends of mRNA resulting in slower degradation, storage and translation inhibition (Huarte *et al.*, 1992). In contrast to sperm cells, which accumulate protamine mRNA to keep translating the mRNA after nuclear condensation (Zhong *et al.*, 2001), the oocyte can store mRNA without any active current translation (Huarte *et al.*, 1987).

Molecular oocyte maturation

Even though our subject of study is large compared with other species, the general problems faced are related to very small amounts of material, very little information on bovine sequences and very little information on the genes specific to the oocyte.

First, there is very little information on specific bovine sequences in any tissue. Most DNA banks contain mainly human and mouse sequences as far as mammals are concerned. Fortunately, when bovine sequences are found through differential display (DDRT) or suppressive subtractive hybridization (SSH), a significant proportion, 53% for oocytes and 21% for granulosa cells (Robert *et al.*, 2000, 2001), can be matched to related sequences in humans or mice. Naturally, there are many more sequences known from somatic tissues, particularly in humans, than from germinal tissues. Therefore, when a given clone is unmatched in available records it does not immediately follow that it is totally new. Indeed, using DDRT or SSH, most of the sequences obtained are the 3'-untranslated region (3'-UTR) and a small portion of the coding region if the clone is long enough. Sometimes the sequence analogy is found with an uncharacterized piece of DNA. Expression sequence tags (EST) are often obtained through massive sequencing of libraries with little information on the coding versus non-coding regions. A strong or long analogy with a given EST might be useful if the sequence present in Genbank is longer than that obtained from the bovine analysis. In this case, new primers can be made to amplify the bovine gene and further sequencing can reveal whether the homology is maintained over the rest of the clone.

A number of cDNA arrays are becoming available for both humans and mice. Most, if not all, of these arrays contain ESTs which, as mentioned above, are not very useful for analytical purposes. In contrast, the known genes may lead to rapid identification of clones. The coding regions of most genes are quite well conserved across mammals and most hybridizations will reveal a conserved sequence. Other types of array, such as the oligo arrays, are much less useful for identifying cross-hybridization events. The length of the DNA sequence on the slide is often between 20 and 30 bp and requires precise hybridization between homologous tissues. Our experience with nylon arrays (Clontech) made for human housekeeping genes or apoptosis genes hybridized with cDNA from bovine oocytes or cumulus cells indicates that cross-species assays will be helpful for identifying a number of obvious genes in species for which the information is more limited.

Second, in addition to the limited amount of information available from the DNA databank relevant to ruminants, the material available from oocytes and embryos is extremely limited and this is true for all mammals and is quite marked for humans. This limitation has retarded quite significantly the research progression on gene analysis and function in the early part of life. Fortunately, new tools will soon be available both in the genomic and the proteomic families.

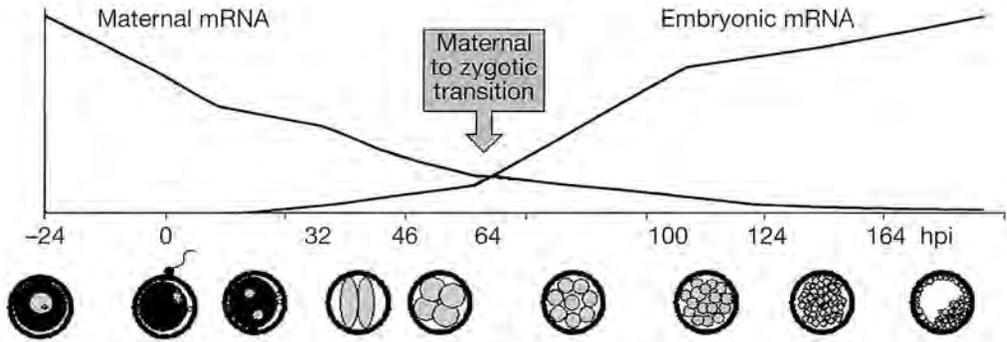


Fig. 2. Expression of mRNA of maternal and embryonic origin during the first steps of bovine embryo development. hpi: hour post insemination.

RNA quantities

It is well known that during oogenesis, the oocyte accumulates RNA to drive the early stages of embryonic development with very little transcription (Bachvarova, 1992; Plante *et al.*, 1994). In cows, which are different in this respect from mice, the resumption of transcription by the embryonic genome becomes apparent at the 8- to 16-cell stage (Memili *et al.*, 1998). Therefore, the oocyte has to accumulate information in the form of stable mRNA. The stabilization of mRNA that allows its storage and prevents its degradation is dependent on *cis*-acting elements in the 3'-UTR interacting with the 5'-end of the mRNA (Mendez and Richter, 2001). Nevertheless, in mice, in the hours after the beginning of maturation, the amount of mRNA decreases sharply (Bachvarova *et al.*, 1985). Consequently, the amount of mRNA available for study is very small and decreases progressively from the beginning of maturation to the maternal to zygotic transition (Fig. 2).

PCR enables the amplification of minute amounts of material as available in oocytes and embryos. The standard protocol of subtractive hybridization requires a few micrograms of poly(A) RNA rendering the use of this technique useless with rare tissues. Thus, using the PCR based techniques, only a few oocytes are required and, as the oocytes in the ovary form a heterogeneous group, it enables selection from precise and defined subgroups of oocytes, for example oocytes from follicles < 2 mm in diameter (low competence), from follicles 3–5 mm in diameter (medium competence) and from follicles > 5 mm in diameter (high competence) (Blondin and Sirard, 1995).

mRNA extraction

Working with oocytes and embryos often requires that small pools of samples are made which results in very small amounts of mRNA; therefore, it would be useful to have an internal standard to assess mRNA recovery. Exogenous control mRNA (rabbit globin) is often added before the extraction to account for the variations caused by the manipulation of the samples (De Sousa *et al.*, 1998; Wrenzycki *et al.*, 1999; Ma *et al.*, 2001). However, the rabbit globin mRNA produced commercially is extracted from reticulocytes and contains up to 20% (according to the manufacturer) of contaminating mRNAs, possibly including some housekeeping genes that are used as reference in our real-time PCR assays. Therefore, care must be taken and appropriate controls must be made to ensure that this practice does not influence the quantification of the gene of interest. With regard to this concern, we made an

exogenous control using mRNA produced *in vitro* for the green fluorescent protein (GFP) and free of any contaminating mRNAs.

RNA quantification

Not only is mRNA present in limited amounts, but also measurement of the relative amount is difficult. Northern blot analysis cannot be used when working with mammalian oocytes or preimplantation embryos because of the difficulty in obtaining the required amount of starting material. It is estimated that about 300 oocytes or 50 blastocysts are required to analyse the expression of a single gene (Watson *et al.*, 1992; Bilodeau-Goeseels and Schultz, 1997). The use of PCR has overcome the problems associated with limited tissue supply and has enabled the efficient detection of specific transcripts. However, the amplification plateau phase with regular PCR results in a more qualitative than quantitative assessment. With real-time PCR, amplification can be monitored at each cycle to assess initial quantity, with much more precision and more sensitivity (Robert *et al.*, 2002). The competitive PCR procedure has been used successfully to measure gene expression in preimplantation embryos (Krussel *et al.*, 1998; Moley *et al.*, 1998; Anderson *et al.*, 1999).

It is clear that the study of gene expression during the preimplantation period requires a better endogenous standard to normalize the RNA expression to justify the variations observed. Robert *et al.* (2002) measured a number of housekeeping genes (Actin, GAPDH, ubiquitin, lamin B, tubulin, histone H2a, U2snRNA, fibronectin and 18S rRNA) from the GV stage to the blastocyst stage in bovine embryos *in vitro* using SybrGreen in a real-time PCR experiment (LightCycler, Roche). The data were taken above the temperature of the primer dimers so that they did not interfere with the results. The results indicated that mRNA expression of the different housekeeping genes had profiles similar to the maternal RNA degradation pattern present in cows. As the embryonic genome is almost transcriptionally inactive during the first few cell divisions, the expression of mRNA decreases until major resumption of transcription occurs (Memili *et al.*, 1998). To our knowledge, among the housekeeping genes selected, only U2snRNA mRNA expression has been studied during the bovine preimplantation period (Watson *et al.*, 1992) and it was concluded that the amount of transcript was constant until the 8- to 16-cell stage and was followed by an increase in RNA expression at the blastocyst stage, which corroborates our results. From all the candidate genes studied, only histone H2a mRNA expression was constant throughout the preimplantation period. This finding highlights an important issue that the definition of a good endogenous standard remains to be clarified. Should the relative standard be a gene that shows a normal expression pattern or one that shows no variation in its mRNA expression?

Comparing mRNA in two populations of oocytes

One objective of our studies is to identify mRNA associated with oocyte maturation and embryonic developmental competency. Therefore, Robert *et al.* (2000) used bovine ovaries recovered from an abattoir and collected the oocytes from two follicle size categories (<2 mm in diameter and 3–5 mm in diameter) to evaluate the differentially expressed mRNAs. Two different technical approaches, the DDRT technique and SSH, were used to compare the mRNA pools of the oocytes. The DDRT technique was optimized to use a small number of oocytes ($n = 10$) without RNA extraction. Triplicates of each follicle size were used to minimize the variability within group sizes. Because of the relatively variable band patterns, bands were considered to be differentially present or absent between follicle size groups only if the band was present or absent in two of the replicates. Out of the 24 series of DDRT comparisons, only 28 bands were considered and after sequence analysis, three clones showed

strong homology with pregnancy-specific glycoprotein, *Drosophila* lethal giant larvae protein and surfactant protein B. With the DDRT technique, the average clone size was 150–200 bp and all the sequences were located in 3'-UTR next to the poly(A) tail. With the SSH technique, we made the two subtractions, large minus small and small versus large follicles. The size of the insert ranged from 200 bp to 1.2 kb, with an average size of 550 bp. Before characterization of the clones, a differential hybridization procedure was performed to identify the false positives. From the large minus small subtraction, 450 clones were studied, only 69 of which were revealed to be true positives. However, for the reverse subtraction, only two clones out of 96 were found to be true positive. Several known genes, such as cyclin B1, splicing factor ccl.4, cytochrome C oxidase and mineralocorticoid receptor, were identified, whereas numerous other clones remain unidentified. Our analysis revealed that the SSH resulted in longer clones than DDRT and showed high specificity.

Expression of specific genes in bovine oocytes

Once interesting genes have been identified through library subtraction or the candidate gene approach, a precise measurement method is required. As mentioned above for the housekeeping genes, real-time PCR is a fairly reliable and functional technique for measuring mRNA contents in a pool of ten oocytes or embryos and can be applied to answer questions such as does the mRNA content vary from the two- to eight-cell stage or according to the size of the follicle of origin, the male used or even according to the culture media? Some of these questions have started to be answered. A few groups have studied a number of genes related to cloning (Wrenzycki *et al.*, 2001), glucose transporter (Augustin *et al.*, 2001) and growth factors (Watson *et al.*, 2001; Yaseen *et al.*, 2001) using mainly semi-quantitative PCR. Nevertheless, this technique allows the proposal of hypotheses concerning upregulation or downregulation of these genes during early development. However, the problem of interpretation is then encountered. It cannot be assumed that there is a direct correlation between mRNA and protein expression (Gygi *et al.*, 1999). An increase in an RNA transcript does not necessarily implicate an increase in the translation of the corresponding protein. Many changes in specific RNA content detected in preimplantation embryos may have no developmental consequences and it is difficult to discern which are functionally significant. In this situation, proteomics becomes a very useful tool.

The poly(A) tail

The method normally used to quantify gene expression is coupling the conversion of mRNA into cDNA using reverse transcriptase with PCR amplification of a specific gene. There are at least three ways to synthesize first-strand cDNA (reverse transcription) from the total RNA extraction by using different oligonucleotide primers. It is usual to add a poly(dT) oligonucleotide to hybridize on the poly(A) tail of mRNAs. An alternative method is specific priming using an oligonucleotide unique to the coding region of a known gene. The other approach is to use hexamers or nonamers (small randomized six or nine nucleotide oligos) which will hybridize at random on all RNAs including non-polyadenylated ribosomal, histone and transfer RNAs.

Maternal mRNAs in eukaryote oocytes can be divided into two main classes. The first class contains stored mRNAs with relatively short poly(A) tails that are in a dormant translation state (Bachvarova, 1992). There is evidence that stored maternal mRNAs do not bind efficiently to oligo(dT)-cellulose (Dworkin *et al.*, 1985). In the second class, the maternal mRNAs are not stored and possess long poly(A) tails associated with translation during oocyte growth

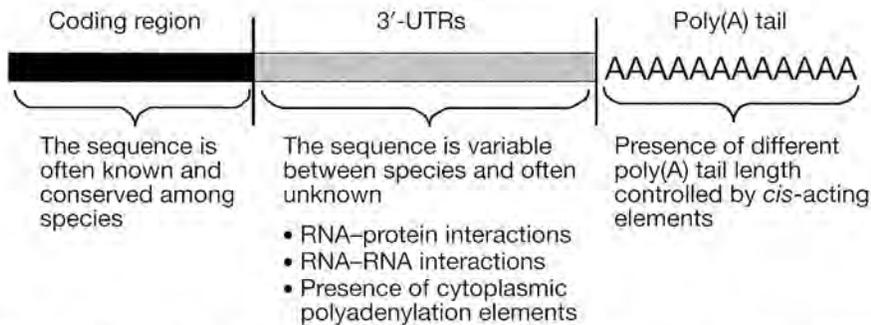


Fig. 3. Translation of maternal mRNAs synthesized during oogenesis is controlled by the elongation or shortening of its poly(A) tail. This specific pattern is dependent on the presence or absence of *cis*-acting elements located in the 3' UTR.

that are shortened during oocyte maturation (translation silencing) by a default deadenylation mechanism (no specific sequences required) (Varnum and Wormington, 1990; Bachvarova, 1992). The initiation of translation of dormant maternal mRNAs is associated with cytoplasmic poly(A) tail lengthening at meiotic maturation or at a specific time after fertilization (Wickens, 1990) and is regulated by *cis*-acting elements (Fox *et al.*, 1989; McGrew *et al.*, 1989; Oh *et al.*, 2000) and *trans*-acting factors (Mendez and Richter, 2001) (Fig. 3). Translation initiation of stored mRNAs is blocked at the initiation step by specific protein interactions between their 3'- and 5'-ends (Stebbins-Boaz *et al.*, 1999). During oocyte maturation in cows, some maternal transcripts seem to follow the default deadenylation pathway (Brevini-Gandolfi *et al.*, 1999). Even though not proven in that species, the translational control of stored maternal mRNAs by cytoplasmic polyadenylation probably occurs as this mechanism seems to be evolutionarily well conserved (Gebauer and Richter, 1996; Verrotti *et al.*, 1996; Ezzeddine *et al.*, 2002).

mRNAs that lack a poly(A) tail are reverse transcribed by adding random nonamers (or hexamers) to prime the reaction. However, the poly(A) tail of the mRNA would be expected to be available still for annealing with the oligo(dT) even in the presence of the random nonamers. However, our experiences indicate that a higher quantity of nonamers out-competed the oligo(dT) resulting in poor cDNA amplification. The results from our studies showed that using oligo(dT) is the preferred technique. We obtained an augmentation of up to 18-fold in the RNA expression of genes when using oligo(dT) instead of nonamers in the same conditions. The only exception was with histone2a which lacks a poly(A) tail. However, oligo(dT) primed to small A-sequences resulting in an amplification that was fivefold lower than with nonamers (M. A. Sirard, I. Dufort and C. Robert, unpublished).

mRNA limitations

In normal somatic cells, increased expression of a given mRNA is normally associated with increased protein expression and increased function. In somatic tissue, an increase in mRNA can be further confirmed by western blot analysis, but in oocytes or embryos, a large number of cells are required to perform western blot analysis. Levesque and Sirard (1996) used up to 300 oocytes to reveal the presence of a relatively abundant protein, cyclin B. Alternatively, MSY2, which is very abundant, can be detected with only 50 mouse oocytes (Yu *et al.*, 2001). An alternative solution is to use immunohistochemistry; however, very specific and sensitive antibodies are required to confirm the presence of the protein and quantification remains difficult. Antibodies are often available for proteins that are also present in large amounts in other tissues. This is often the case for proteins involved in cytoskeleton, housekeeping, sugar

metabolism, trafficking, but many important proteins might be specific to the oocyte and not present in other tissues. For example, the oocyte is the only cell that is known to be capable of complete nuclear reprogramming and must have a number of genes specific to that function. It is possible that some genes involved in the reprogramming system are in sufficient amounts to be detected by PCR, but not by antibodies.

Polysome fractionation

In maturing mammalian oocytes and during early embryonic development, cytoplasmic polyadenylation of stored maternal mRNAs is a well-known mechanism responsible for the onset of protein synthesis (Huarte *et al.*, 1987; Paynton *et al.*, 1988; Vassalli *et al.*, 1989). Moreover, the poly(A) tail length of stored maternal mRNAs can be partially shortened and subsequently lengthened to its original size without apparent mRNA degradation (Oh *et al.*, 2000). As post-transcriptional regulation of translation is involved, the maternal mRNA expression provides little information if the mRNAs are translated into their cognate proteins. A possible solution to this problem is to use mRNA extracted from polysomal fractions (ribosome loading) that reflect active translation. It is possible to couple polysomal mRNAs isolated by sucrose density gradient fractionation with quantitative RT-PCR to study regulated recruitment of maternal mRNAs during embryonic genome activation (Wang and Latham, 2000) or oocyte maturation. Even more interesting, it was shown that mRNA isolated by polysome fractionation could be used in DNA arrays to confirm that the mRNA amount corresponded to the expression pattern (Johannes *et al.*, 1999; Zong *et al.*, 1999).

DNA arrays

Making expression libraries or using sub-fractions of them by selective subtractions produces a large amount of potential information (Fig. 4). This information has often to be validated for tissue specificity if, for example, two tissues are subtracted or for treatment effect if two different conditions are used. It is possible to hybridize the subtracted library to both initial libraries to verify the number of false positives, which can be achieved by labelling the initial libraries to hybridize Southern blots made with dots of the selected clones. The false positives are not rare and sometimes they represent more than half of the subtracted library (Robert *et al.*, 2001). The traditional hybridization on nylon is being replaced by glass arrays that allow several to hundreds of similar arrays to be made.

One of the main problems with the use of DNA arrays for oocytes or embryos is still the limitation of material to hybridize. The clones on the array can be amplified but to assess a gene expression profile for embryos from one culture system compared with another, the material is limiting again. There are ways to amplify the mRNA before or instead of making a cDNA library. Our experience with T7 linear amplification of the RNA did not result in the expected 2000-fold yield increase as reported by Phillips and Eberwine (1996). However, the mRNAs amplified have shown a relative abundance of the transcripts between the samples as the mRNA expression profiles were the same with or without pre-amplification. Thus, if comparisons are to be made across the pre-implantation period for a specific gene, the T7 amplification system could be used, but this technique is not as reliable for the comparison across genes.

Requirement for protein analysis

As mentioned above, when mRNA expression increases or is higher than a different sample or treatment, the value of this information is questioned. Of course some RNAs are never

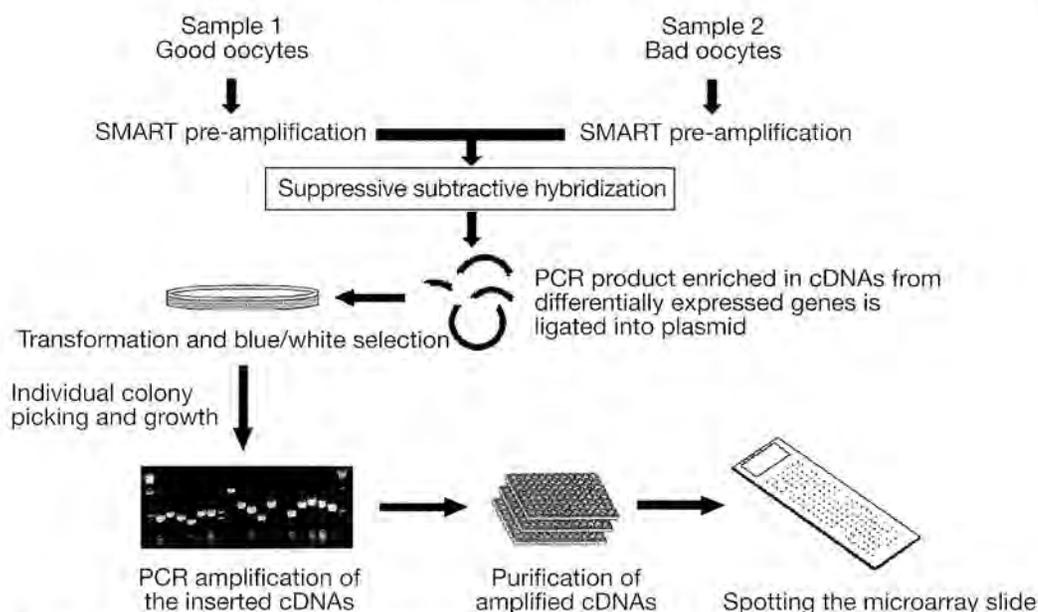


Fig. 4. Example of the possible steps implicated in making a microarray. The two pools of oocytes are first amplified with the PCR-based SMART technique (Clontech) and go through suppressive subtractive hybridization. The differentially expressed cDNAs are ligated into an expression vector, transformed and amplified. The purified PCR is then spotted on to microarray slides.

translated and have an intra-nuclear function but these would normally be lacking a poly(A) tail and would not be recovered in the RT-PCR using poly(dT) oligos or the resulting subtractive libraries. Two methods are becoming sensitive and precise enough to assess function: proteomics and RNA interference (RNAi).

Proteomics

The systematic analysis of protein patterns is not a new technique (O'Farrell, 1975), but is becoming more sensitive and more reliable because of better separation methods and mass spectrometry analysis. Although two-dimensional gel electrophoresis analysis has been used for more than two decades for mammalian oocytes and embryos, the information relative to specific gene expression remains limited. New studies are being reported, for example by Coonrod *et al.* (2002), who used two-dimensional gel analysis to begin building a mouse oocyte proteomic database, and have resolved and digitized over 500 silver-stained proteins to date. Coonrod *et al.* (2002) used cell-surface labelling with biotin to identify a subset of 80 putative egg surface proteins. Amino acid microsequences from over 30 of the surface-labelled proteins have been obtained by tandem mass spectrometry. Sequences from eight of these proteins do not match any sequences from protein and DNA databanks, indicating that these proteins are novel. Their major current research goal is now to clone, characterize and express the novel proteins that are shown to be specific to the ovary and to investigate their functional roles in sperm-egg interaction.

We are pursuing similar characterization in our laboratories but instead using radio-labelling of oocytes or embryos at different stages or from different source or treatments to assess the oocyte competence to specific proteins. The radiolabelling approach with cysteine

and [³⁵S]methionine allows us to identify between 300 and 600 proteins with groups of 50 or fewer oocytes. With silver nitrate staining, a similar number of proteins can be obtained with 200 oocytes. A high capacity electrophoresis unit coupled with a computer analysis system allows a quite useful repeatability and the definition of specific patterns. So far we have characterized the patterns of protein synthesis at 4 h intervals during the 24 h of maturation in bovine oocytes and in fast cleaving two-cell embryos versus slow cleaving two-cell embryos in cows (L. Massicotte, K. Coenen and M. R. Sirard, unpublished). Identification is required to relate this information to the subtracted libraries mentioned before, for identifying proteins on a gel. Two options are available. The first option is traditional western blot analysis which is limiting as the proteins have to be known, and which requires hundreds of oocytes, the corresponding antibody and a new gel for each protein studied. In the case of unknown proteins, the second option is the protein gel extraction and mass spectrometry analysis. The technology to extract proteins from a two-dimensional gel for mass spectrometry by matrix assisted-laser desorption/ionization–time of flight (MALDI–TOF) or mass spectrometry–mass spectrometry (MS–MS) analysis is developing rapidly. The low cost approach still requires the isolation of 10 ng proteins, which correspond approximately to the smallest amount visible on a two-dimensional gel stained with colloidal Coomassie blue (Bio-Rad Laboratories). The required number of oocytes to obtain between 400 and 500 blue spots on a two-dimensional gels is > 2500 (our own observation). The protein of interest (that is, which is expressed differently in two stages or treatments) may then be among the 500 visible blue spots on the gel. Subsequently the sample goes through the identification process. The first and easiest step for identification is the MALDI–TOF technique (Lauber *et al.*, 2001), which consists of digestion of the protein with trypsin followed by measurement of the molecular weight of the individual fragments (Issaq, 2001). The pattern of peptide masses obtained can be compared with known banks. Special care must be taken as the peptide masses can be affected by different levels of methylation or phosphorylation but these deviations can be corrected on some available databanks (<http://genomics.msu.edu/MSSSF/massc-ta.html>). A substantial homology is required to obtain a positive identification and this is quite a limitation for mouse or human specimens. In addition to this limitation, most of the peptide banks are constituted of somatic tissue analysis. Therefore, proteins specific to oocytes and early embryos are not yet available. A possible solution to this problem is the further analysis of some peptides to reveal their amino acid sequences. This is carried out with an MS–MS and the technology is actually becoming more affordable as well as more sensitive to allow the use of smaller samples. Once the amino acid sequence is known for one or two peptides, it is possible to generate degenerated DNA sequences for screening related genes in DNA data banks. If the sequence is still unknown, the degenerated sequence can be used to rescue the new mRNA.

RNA interference

RNA interference (RNAi), the targeted mRNA degradation by small double-stranded RNA (dsRNA), is a useful technique for studying gene function in several organisms. RNAi has attracted considerable attention because it is a means of knocking out the activity of specific genes. The destruction of the targeted mRNA by dsRNA occurs before translation and targets exon sequences; dsRNA directed against intron sequences is ineffective (Fire *et al.*, 1998). An important feature of the mechanism of RNAi is the processing of long dsRNAs into duplexes of 21–25 nucleotide RNAs. Therefore, the 21-nucleotide RNA products were named small interfering RNAs or silencing RNAs (siRNAs). During the RNAi, an siRNA-containing endonuclease complex cleaves a single-stranded target RNA in the middle of the region complementary to the 21 nucleotide guide siRNA of the siRNA duplex (Elbashir *et al.*, 2001).

Despite the lack of profound mechanistic understanding, RNAi has rapidly developed into an important tool for reverse genetics and has been widely applied in *Caenorhabditis elegans* (Fraser *et al.*, 2000; Gonczy *et al.*, 2000), as well as in insects (Lam and Thummel, 2000) and vertebrates (Wargelius *et al.*, 1999). In mammals it was shown that RNAi inhibits maternal and zygotic gene function in early mouse embryos efficiently (Svoboda *et al.*, 2000; Wianny and Zernicka-Goetz, 2000).

Conclusions

The maturation of the mammalian oocyte is about to be redefined by the complexity of the molecular changes that occur during this period. The fine-tuning of gene expression and even more storage of crucial mRNA and proteins are determinants of the future ability of an egg to become an embryo. The limitations inherent to the molecular approaches, namely, the amount of material, the sequences available and the specificity of oocyte gene expression patterns, represent new challenges. Nevertheless, the understanding of this molecular complexity will reveal how the oocyte creates new life at each generation.

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