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Microarray and RNA Interference: the Tools to Understand Gene Expression in Preimplantation Embryo Development

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ABSTRACT

Ultimately, one wishes to determine how genes and the proteins they encode function in the intact embryo. Today, the exploration of gene function often begins with a DNA microarray. This technique has revolutionized the way in which gene expression is now analyzed by allowing the RNA products of thousands of genes to be monitored at once. Comprehensive studies of gene expression and identifying gene interaction partners also provide an additional layer of information useful for predicting gene function. Other approaches to discover a gene's function, include searching for homologous genes in other organisms and determining when and where a gene is expressed. Searching for homologous genes and analyzing gene expression patterns can provide clues about gene function, but they do not reveal what exactly a gene does inside a cell. Genetic engineering provides a powerful solution to this problem especially allows one to specifically produce such gene knockouts. Normally, only one of the two DNA strands in is transcribed into RNA, and it is always the same strand for a given gene. If a cloned gene is engineered so that the opposite DNA strand is transcribed instead, it will produce antisense RNA molecules that have a sequence complementary to the normal RNA transcripts. Such antisense RNA can often hybridize with the "sense" RNA and thereby inhibit the synthesis of the corresponding protein. The purpose of this review is to focus on microarray and RNA interference technology as tools to study gene expression in bovine embryos during the preimplantation period.

Keywords: microarray, RNA interference, embryo, preimplantation, gene expression.

1. INTRODUCTION

In mammals, the meeting of the oocyte and sperm, and subsequent fertilization, takes place in the ampulla of the oviduct. During the following days, the embryo travels down from the oviduct to the uterus, and prepares for implantation. Preimplantation development

can also occur in vitro. In all mammalian species studied, the preimplantation stage is characterized by a relatively synchronous doubling of cell numbers until the 8-cell stage followed by asynchronous cell divisions after compaction. At the 8- to 16-cell stage the embryo enters the uterine environment, develops into a blastocyst, in which the first events of cellular differentiation are observed. At the blastocyst stage the embryo hatches from the surrounding zona pellucida and subsequently implants in the uterus.

What do we know about this mechanism that activate gene expression in mammals and thereby turn on the developmental program? Historically, answers to this question have relied heavily on studies done with fertilized-oocytes from frogs and flies [1] In mammals, the preimplantation embryo is defined by the development of the zygote through several cleavage divisions, the activation of embryonic transcription, and the morphogenetic events of compaction and cavitations resulting in the formation of a blastocyst. The period from fertilization to implantation involves various morphological, cellular, and biochemical changes related to genomic activity [2]. Various technologies, such as artificial insemination, embryo transfer, and cloning, have been applied to bovine reproduction [3,4]. Precise knowledge of the gene expression profile during preimplantation is necessary to reduce early losses and to improve the reproductive efficiency of these new technologies [5-7]. However, little is known about the complex molecular regulation of embryos and extra-embryonic membrane development in cattle. Thus, the genes to be profiled include new, functional gene candidates. This suggests an assessment method for key genes to help clarify the complex mechanisms in early embryo development, including also trophoblast cell proliferation and differentiation.

2. DEVELOPMENTAL COMPETENCE

Developmental competence is the ability of the oocyte to produce normal, viable and fertile offspring after fertilization. The developmental competence of the oocyte is acquired within the ovary during the stages that precede ovulation or in case of in vitro maturation (IVM), precede the isolation of the oocyte from its follicle [8]. Oocyte competence is a difficult parameter to assess since embryonic development may fail due to other reasons independent of oocyte quality. Developmental competence is usually expressed as the percentage of oocytes that can develop to the blastocyst stage [9]. However, development to the blastocyst stage does not guarantee that the embryo will develop to term. Other aspects used to evaluate developmental competence include morphological evaluations, such as number of blastomeres or the ratio between inner cells mass (ICM) and trophoectoderm (TE) cells number and metabolic rates [10]. The size and the quality of the follicle of origin [11] influence the developmental capacity of bovine oocytes. It appears that oocyte requires an additional prematuration to express their competence [12]. If in vivo, this prematuration occurs during preovulatory growth before the lutenising hormone (LH) surge, the ovarian morphology, the number and size of the follicles present in the ovary at the time of aspiration, the composition of the follicular fluid [6,11,13] may be critical for the oocyte to acquire developmental competence. The developmental competence of the oocyte may also be lost during IVM since the number and quality of cumulus cells surrounding the oocyte are important in this process [9,14].

The absence of reliable markers for the identification of viable embryos for transfer at the early cleavage stage is likely to contribute to the generally low implantation rates in in vitro fertilization (IVF) treatment [15]. Early

cleavage is an indicator of increased developmental potential in embryos and may be useful as a criterion in the selection of embryos for transfer. To improve the selection of the embryo with the highest implantation potential, Van Montfoort et al., 2004 [16] suggested that selection for transfer should not be based on cell number and morphology on the day of transfer alone but also on early cleavage status.

2.1 Preimplantation Embryo Development

The changes during mammalian preimplantation development include the inside zona diameter of gamete from less than 30 μm in the primordial follicle to more than 120 μm in the bovine tertiary follicle [17] and also the elongation of embryonic tissues, cell-cell contact between the mother and the embryo, and placentation. The embryo begins to form the placenta around day 20 of gestation in the bovine [18,19], while embryonic trophoblast and endometrial cells tightly unite to form placentomes on day 30 [20,21]. Embryonic cells undergo both proliferation and differentiation to form the fetus and placenta throughout early embryogenesis. Reprogramming of the genome may be completed and reset during these steps, with embryonic development progressing to temporal and spatial gene expression [22,23].

2.2 Gene Expression in Preimplantation Embryo

Gene expression (also protein expression or simply expression) is the process by which a gene's information is converted into the structures and functions of a cell. Gene expression is a multi-step process that begins with transcription and translation and is followed by folding, post-translational modification and targeting. The amount of protein that a cell expresses depends on the tissue, the developmental stage of the organism and the metabolic or physiologic state of the cell. Gene expression is one of the most important principles underlying the development and control of cells, systems and organisms. Essentially gene expression is the process by which genetic information is converted into entities (mainly proteins) that contribute to the structure and operation of a cell. The study of gene expression not only encompasses the transcription of DNA to RNA (transcription) by predominantly messenger RNA (mRNA), but also transfer RNA (tRNA) and ribosomal RNA (rRNA), followed by protein synthesis (translation). Initiation of transcription is the most important step in gene expression. Without the initiation of transcription, and the subsequent transcription of the gene into mRNA by RNA polymerase, the phenotype controlled by the gene will not be seen. Therefore in depth studies have revealed about what is needed for transcription to begin. In conjunction with the activation of the embryonic genome, conventional one dimensional of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has shown that major changes occur in protein synthesis between day 1 (2-cell stage) and 2 (4- to 8-cell stage) of preimplantation mouse embryo development [24,25]. The first proteins synthesized in the late 2-cell embryos coinciding with embryonic genome activation appear to be heat shock proteins (67,000-70,000 daltons) [26]. During the late 4- and 8-cell stage new transcription is necessary to prepare the embryo for compaction, while during the morula to blastocyst transition there is also a change in transcriptional activity in line with the increase in the rate of protein synthesis [27]. These changes ultimately lead to the appearance of tissue or stage specific polypeptides in the ICM and TE cells at the

blastocyst stage [28,29].

Early embryonic development of most animals requires a specific complement or abundance of oogenetic mRNAs and proteins to confer full developmental competence following fertilization [30,31]. The formation of zygotes with successful fertilization triggers cohorts of events that begin by repeated cycles of cell division, activation of embryonic genome, compaction and differentiation into ICM and TE cells resulting in the formation of blastocyst [32-34]. While these series of events have been the distinctive characteristics of preimplantation embryo development, temporal occurrences vary between different species [33,35] and relies on the sequential and temporal expression of about 10,000 genes out of which the sequence, expression and function of only a very minor portion has been recognized so far [34,36]. Generally, the control of development in preimplantation embryos is guided by two major activation events occurring temporally at the preimplantation development stage. These are maternal and zygotic gene activation events that control development sequentially.

2.3 Maternal Gene Activation and Development Control

Early embryonic development in mammalian species is regulated by maternal transcripts. As a result, any activity that requires the creation and development of an embryo, whether it is in the context of infertility treatment or in the creation of a reconstructed embryo by nuclear transfer, is dependent on the intrinsic ability of that oocyte to support development [37,38]. This is, however, guided by a very stable form of RNA that accumulated in the oocyte and translated during maturation, fertilization and early embryonic development [39]. These translationally dormant mRNAs encode a variety of products which are activated in a stage and sequence specific manner in early development [39,40]. During this time, different factors were mentioned to be responsible for the regulation of translation in this stored mRNAs. Sub-cellular localization, cytoplasmic polyadenylation, and Y-box proteins have emerged as leading candidates to regulate the translation of maternal mRNAs [41]. Although these and a number of other mechanisms are probably responsible for the translational control of maternal mRNA, one that appears to be widespread is cytoplasmic poly (A) elongation [40,42,43]. As a result, mRNAs and proteins synthesized and stored during oogenesis initiate and support a developmental program induced by sperm penetration [6,44-46]. However, after one to four cleavage divisions, based on the species, the maternal phase gradually looses its development control [32,35,47]. This transition from maternal to embryonic control of development is characterized by a degradation of maternal RNA and protein, arrest in embryonic development, increased sensitivity to transcriptional inhibitors such as alpha-amanitin, a burst of transcriptional activity from the embryonic genome [48] and the replacement of transcripts previously degraded and the generation of new transcripts that were not present in the oocyte [49].

2.4 Embryonic Gene Activation and Development Control

In all species, the development beyond early cleavage divisions is dependent on zygotic gene activation and subsequent maintenance of temporally and spatially appropriate zygotic transcription[50]. The trigger for the initiation of embryonic transcription remains unclear [51,52]. However, it involves the synthesis of proteins, which are about 40 proteins in mouse [53].

During the transition from maternal to embryonic control of development, maternal transcripts are depleted and embryo specific transcripts involved in early embryogenesis are generated [54]. The transcription of the 18S, 5.8S, and 28S rRNA polymerase I and their subsequent processing lead to the formation of a distinct nuclear structure of the nucleus [46]. Furthermore, the transition is accompanied by modifications in chromatin structure and post-translational modifications of the transcriptional abilities in early embryos [45]. In addition, a dramatic reprogramming of gene expression occurs during this transition, and this is similar to the molecular foundation for transforming the highly differentiated oocyte into the totipotent blastomeres of the early cleavage stage preimplantation embryo [51]. The timing of zygotic gene activation, or competence to sustain appreciable transcriptional activity in bovine embryos may be controlled temporally by a time-dependent mechanism referred to as zygotic clock rather than by developmental stage [47,55]. This was confirmed by transfecting a reporter gene into 1 cell stage bovine embryos and examines the expression at a particular stage [47]. Similar test has also been done with mouse zygote [55]. In bovine embryos, zygotic gene activation has definitely occurred by the 8- to 16- cells stage as evidenced by incorporation of [3H]-uridine into nuclei and nucleolei at the 8-cells stage [32]. This activation is responsible for controlling subsequent development, and different transcripts are expressed in a stage specific manner. However, first transcript initiation at 2- to 4- cells stages was observed in bovine embryo development and this initiation is ±- amanitin insensitive and is not required for the progression of embryonic development to advanced preimplantation stage.

3. TRADITIONAL METHODS OF GENE EXPRESSION ANALYSIS AND LIMITATIONS

Details on the traditional methods of gene expression analyses have been mentioned elsewhere [56,57]. Traditionally, studies measuring the differences between cell types or cell pathways following treatment or perturbation of the cell have been carried out at the level of transcribed mRNA using methods such as the classical and most well established northern blot [58], RNase protection assay [59], differential display [57], representational difference analysis [60,61] and suppressive subtractive hybridisation [62-63]. These methods, although fruitful and still in use, have limited scope in terms of the number of genes that can be analysed, and some of these methods identify only known genes [56]. As a consequence, genes were not exhaustively characterized for different physiological parameters. The case for developmental studies was not different from this general truth. Techniques are continuously being developed in terms of technical efficiency, cost, sample requirement, and number of samples analysed at a time, albeit the previous methods are valuable and still in use. Recently real-time RT-PCR method has been introduced as an additional tool for gene expression studies. However, it is not a method of choice to explore the expression patterns of multiple genes simultaneously. Therefore, all these techniques have now been superseded by technologies that allow the simultaneous analysis of multiple genes. More robust methods, aiming at capturing gene expression at genomic scales, including serial analysis of gene expression (SAGE) [64-66], expressed sequence tags (EST) library sequencing [67,68], massive parallel signature sequencing (MPSS) and microarray have taken their place [69]. The common trait among these technologies is their capability to capture comprehensive biological information, in which all endpoints

are, measured simultaneously [70]. However, microarrays are more preferred in terms of number of genes that can be analysed simultaneously, ease of controlling and handling the experiment [71].

4. STUDY THE GENE EXPRESSION DURING PREIMPLANTATION EMBRYO USING MICROARRAY AND RNAI

To understand the development of preimplantation embryo, the expression of gene(s) controlling this process has been intensively studied, especially with the development of powerful techniques such as microarray and RNAi. While microarray is used as a tool to measure the expression levels of thousands of genes simultaneously, RNAi can be applied to have further knowledge on the functions of target genes derived from the microarray.

4.1 Microarray as a Method of Gene Expression Analysis

Microarrays are, in principle and practice, extensions of hybridisation-based methods such as southern blot [72], northern blot [58], and dot blots [73], which have been used for decades to identify and quantify nucleic acids in biological samples [74-76]. It is simply a large scale dot blot [77]. However, the main distinction is in the use of an impermeable, rigid substrate, such as glass in microarray experiment [78]. The technique exploits the capacity of nucleic acid strands to recognize the complementary sequences through base pairing [79] and was first developed and introduced by the pioneering works of Schena and colleagues [80]. The near completion of human genome sequence, aggressive identifications of ESTs in different species, and gene expression studies at various laboratories have marked today's genetic era by so many genes whose expression patterns and biological functions need to be investigated yet [71,81,82]. As a consequence of this burgeoning interest, the field of functional genomics has arisen, which encompasses the development and application of methods to examine the expression of large numbers of genes using a holistic approach, rather than on a gene by gene basis (81, 83). Moreover, the value of these tomes of information will be fully realised when the function and control of genes, and their pathways are elucidated [77]. Microarrays have been evolved at this cross road as impetus for solving such numerical mayhem by providing insight in to gene functions using a systematic global approach. Generally two types of microarrays are recognized; cDNA and oligonucleotide arrays and both are sensitive in identifying regulated transcripts [84,85]. A typical microarray expression profiling experiment involves the hybridisation of a fluorescently labelled cDNA probe to cDNA clones or oligonucleotides immobilized onto glass slides and the data offer an insight in to the transcriptional responses of a genome to a particular mutational event or environmental insult [86]. On the other hand, array based technologies, while seductive can sometimes be corruptive [87]. Although the high-throughput technology enables researchers to study expression for thousands of genes simultaneously, experiments by using microarrays may be costly, time consuming, and output is subject to substantial variability [88,89], needs large amount of RNA [80] and the level of mRNA expression does not always reflect protein concentration. Microarrays are also closed systems that enable the investigator to look at the expression of predetermined genes of interest spotted on the glass slide [77]. Furthermore, the generated data are not end point of the study but needs to be confirmed by independent methods. Finally, cDNA arrays are liable for cross-hybridization due

to sequence homology, particularly when several members of the same family are included [90].

4.1.1 Applications of Microarray in Preimplantation Embryo Development

The use of DNA microarrays to examine mammalian development is a small but rapidly growing field of study. It is currently dominated by the exploitation of arrays to perform screens for molecules involved in particular developmental processes [86]. However, addressing the functional pathways and how cellular components work together to regulate and carry out cellular processes, requires the quantitative monitoring of the expression levels of very large numbers of genes repeatedly and reproducibly, under the influence of genetic, biochemical and chemical perturbations [71,79,88]. Oocytes and preimplantation stage embryos express a wide variety of genes of which many have overlapping or apparently redundant functions. Thus, characterisation of preimplantation development at the molecular level in all species requires the simultaneous examination of the full repertoire of functionally related genes [91]. So far, the application of microarray technology to study preimplantation stage embryo development has been hampered by both cultural and technical issues [86]. The frequently mentioned technical problems are the need for large amount of input RNA, which can not be secured from standard embryo sample [88] and by lack of appropriate cDNA collection [92]. On the other hand, the cultural issue refers to the familiarity of developmental biologists with the previous techniques and lack of familiarity with microarrays [86]. As a result, only limited studies were published so far.

Recently, cDNA microarray has been applied successfully to profile the expression pattern of a large number of transcripts involved in various developmental pathways. These include study in the analysis of preimplantation stage human embryos [93,94], study the gene expression in mouse embryos using cDNA arrays [92,95], study bovine oocyte maturation using heterologous cDNA array [96], identification of important genes in oocyte library important for embryogenesis [97], compare the developmental competence between oocyte and preimplantation embryo in bovine [98,99], compare the expression profiles for embryos derived from different origins [100], detect the gene expressions during bovine embryogenesis and implantation [101] and recently identification of differentially expressed genes in preimplantation embryos produced by nuclear transfer to study the effect of this new technology on reprogramming the genes required for development [102,103]. Nevertheless, recently the methodologies have made a remarkable progress and RNA amplification coupled with gene specific target production is a common procedure to pursue microarray experiment, and fill the previous research gaps.

4.1.2 Post Analysis Follow-up and Validation of Microarray Experiments

Although there has been great progress in measurement of gene-expression profiles using microarray technology, investigators are still confronted with a difficult question after completing their experiments: how to validate the large data sets that are generated? There are two approaches to independent confirmation of microarray data: in silico analysis and laboratory-based analysis [104]. The in silico method compares array results with information available in the literature and in public or private expression databases, and provides the opportunity to validate data without further experimentation [105,106]. Agreement between array results from other

groups, as well as with known expression information in the literature, validates the general performance of a system and provides confidence in the overall data, including the unique and novel discoveries made in a study [104]. Laboratory-based validation of data provides independent, experimental verification of gene-expression levels, and typically begins with the same samples that were studied in the initial array experiment(s). The methodology used varies depending on the scientific question, but commonly used techniques include semi-quantitative reverse-transcription PCR (RT–PCR), real-time PCR, northern blot, ribonuclease protection assay, and in situ hybridization or immunohistochemistry using tissue microarrays [96, 103,105,107,108]. Real-time RT–PCR is the choice of many for quantitatively measuring specific mRNAs as, once established, the method is rapid, relatively inexpensive and requires minimal starting template [109,110]. In addition to validating array results at the mRNA level, it is equally important to evaluate expression levels of the corresponding protein products. The selection of the gene set for follow-up analysis in the laboratory depends on the aim (s) of the study, but is influenced by factors such as the relative difference in expression among the samples, biological function, abundance levels, and availability of appropriate reagents (probes and antibodies).

4.2 RNA Interference or Gene Silencing

Presently, the genomes of various species including the bovine are largely sequenced. Moreover, several studies have been carried out during the last decade to investigate the expression patterns of genes in bovine embryogenesis in response to various culture and treatment conditions [6,111-113]. Despite the fact that the bovine genome has been reported to be completely sequenced, the function of most of the genes is not yet known. Till recently, the function of a specific gene in bovine species has been predicted using knockout experiments conducted in mouse (114, 115). However, these knockout technologies are extremely laborious and need

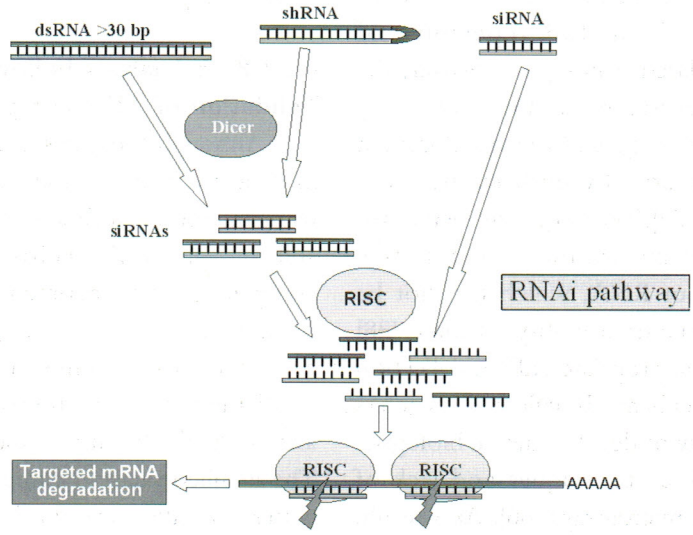

Figure 1. RNAi mechanism in mammalian cell, starts with the processing of double-stranded RNA (dsRNA) or short hairpin RNA (shRNA) into small interfering RNA (siRNA) by the dicer emzyme. Small interfering RNA serves as guide sequences for RNA induced silencing complex (RISC), which recognizes and cleaves the cognate mRNA.

long time to see the effects. So what is needed is a technique that can be used to jump directly from sequence to function in the whole animal. For this, the post transcriptional gene silencing (PTGS) by double-stranded RNA (dsRNA) or RNA interference (RNAi), has emerged as a new tool for studying gene function in an increasing number of organisms [116]. To overcome this, the RNAi approach through introduction of sequence specific dsRNA into the cells has been reported for the first time in Caenorhabditis elegans as an effective tool to study gene function in this species [116]. Due to its relatively easy application and its effectiveness, this technique has been used to study gene function during early embryogenesis in mammalian species including mouse [117-130], swine [131,132] and bovine [133-136]. This approach has been reported to be an effective tool to inhibit genes from both maternal and embryonic genome expressed in those species.

A working model for RNAi is shown in Figure 1. The first step is the production of dsRNA directed against an mRNA. The second step involves the recognition of dsRNA and its processing to produce 21-23 nt siRNAs. The effected step is the recognition of the target mRNA by the siRNAs and the selective degradation of that mRNA. In this section, three mechanistic features of RNAi relevant to the mammalian pathway will be shown: 1) processing of dsRNA into siRNA; and 2) recognition and cleavage of the cognate

Table 1. The applications of gene silencing in different species.

Phylum	Species	Mechanism	Effector	References
Fungi	Neurospora	Quelling	Transgenes	Cogoni and Macino, 1999 [139]
Plants	Arabidopsis	PTGS	Transgenes	Elmayan et al., 1998 [140]
	Nicotiana	Transcriptional gene silencing	Transgenes, Virus	Furner et al., 1998 [141]
	Pitunia	PTGS	Transgenes	Dehio and Schell, 1994 [142]
Invertebrates	C. elegans	RNAiTranscriptional gene silencing	dsRNA, Transgenes	Fire et al., 1998 [116]; Kelly and Fire, 1998 [143]; Ketting et al., 1999 [144]
	D. melanogaster	RNAi	dsRNAshRNA	Misquitta and Paterson, 1999 [145]; Paddison et al., 2002 [146]
	Paramecium	Homology-dependent silencing	Transgene	Ruiz et al., 1998 [147]
	Trypanosome	RNAi	dsRNA	Wang et al., 2000 [148]
Vertebrates	Danio rerio	RNAi	dsRNA	Wargelius et al., 1999 [149]
	Mus musculus	RNAi	dsRNA	Knott et al., 2005 [122]; Plusa et al., 2005 [150]; Wianny and Zernicka-Goetz, 2000 [129]
		RNAi	siRNA	Haraguchi et al., 2004 [120]
	Sus scrofa	RNAi	dsRNA	Anger et al., 2004 [131]; Cabot and Prather, 2003 [132]
	Bos turus	RNAi	dsRNA	Tesfaye et al., 2007 [133]; Nganvongpanit et al., 2006 [134]; Nganvongpanit et al., 2006 [135] ; Paradis et al., 2005 [136]
	Homo sapien	RNAi	dsRNA	Brown and Catteruccia, 2006 [137]
			shRNA	Rossi, 2006 [138]
			siRNA	Gaur, 2006 ; Rossi, 2006 [138]

mRNA, for more detail see Svoboda, 2004 [126].

4.2.1 Application of RNA Interference to Establish Developmental Gene Function

The most widely used RNAi technology has been in cell culture and in vivo studies aimed at understanding the function of an individual or multiple proteins. The complex and remarkably rapid change that occurs during development of the fertilized oocyte or zygote into an adult organism remains a large mystery. There would appear to be a great potential for RNAi technology to unravel the cellular and molecular events that regulate development processes. Methods for silencing single or multiple selected genes in developing embryos in vivo and in vitro are beginning to reveal the functions of specific proteins in development processes (Table 1). The RNAi was used to demonstrate that siRNAs directed against the mRNA encoding Oct-3/4 and C-mos resulted in depletion of the encoded proteins and phenotypes similar to those observed in Oct-3/4 and C-mos knockout mice [121]. A key role for microtubule-associated protein-2 in the regulation of dendrite outgrowth in developing brain neurons was demonstrated using siRNAs [137]. The transcription factor Myc is known to play a fundamental role in the regulation of cell proliferation. A key role for the novel Myc target gene Mina53 in the regulation of cell proliferation by Myc was demonstrated using RNAi technology [138].

4.2.2 Application of RNA Interference in Preimplantation Embryo

In Table 2, the application of RNAi in mammalian embryos is shown. The first application was reported in 2000 by 2 research groups [126,129]. Wianny and Zernicka-Goetz, 2000 [129] used this technique in mouse oocytes and preimplantation embryos. In this experiment, three genes namely: MmGFP, C-mos, and E-cadherin were tested. For the first gene, a mouse line was created in which carried the MmGFP gene, was paternally inherited to prevent complications from maternal transcripts and translation products. Tests showed that when embryos were injected with dsRNA specific for MmGFP, the fluorescence was significantly diminished; indicating that expression of the gene had been blocked. Also, when the embryos were injected with dsRNA specific for C-mos or E-cadherin, no effect on the fluorescence occurred, although changes resulted from the blockage of these two genes were observed, which indicates that in mice as well as in invertebrates, the interference effect is specific. A similar test was done with dsRNA specific for E-cadherin. The disruption of this gene leads to uncompaction, a severe preimplantation defect, which prevents the embryo from developing correctly [114,115]. Similar effects to the MmGFP study were found, dsRNA specific for E-cadherin resulted in uncompaction of the embryos and dsRNA specific for C-mos or MmGFP did not. The final test involved C-mos, a maternally inherited gene which arrests maturing oocytes at metaphase during the second meiotic division. The injection of dsRNA specific for C-mos caused 63% of the injected cells to fail to maintain arrest at MII, whereas 1-2% of the control group failed to maintain arrest [129]. This demonstrated that, unlike the knockout method, dsRNA can block expression of maternally provided gene products. RNA interference is important because it allows researchers to study the effects of genes loss of their function on developing embryos without the complications of the gene knockout method. The application of this mechanism to vertebrates and then to mammals is likely to provides better models

Table 2. The applications of RNAi in mammalian preimplantation embryos.

Species	Tissue	Gene	Molecule	Reference
Mouse	Oocytes	Bmp-15	dsRNA	Gui and Joyce, 2005 [119]
		C-mos	dsRNA	Svoboda et al., 2000 [128]; Wianny and Zernicka-Goetz, 2000 [129]
		Plat	dsRNA	Svoboda et al., 2000 [128]
		PLCz	shRNA	Knott et al., 2005 [122]
		Egfp	dsRNA	Wianny and Zernicka-Goetz, 2000 [129]; Stein et al., 2003 [124]
		Gdf-9	dsRNA	Gui and Joyce, 2005 [119]
		Itpr1	dsRNA	Xu et al., 2003 [130]
		Miss	dsRNA	Lefebvre et al., 2002 [151]
		Doc1r	dsRNA	Terret et al., 2003 [152]
		Bnc	dsRNA	Ma et al., 2002 [153]
		Ctcf	dsRNA	Fedoriw et al., 2004 [154]
		Msy2	dsRNA	Yu et al., 2004 [155]
	Embryo	Dicer1	siRNA	Svoboda et al., 2001 [127]
		E-cadherin	dsRNA	Wianny and Zernica-Goetz, 2000 [129]; Sonn et al., 2004 [123]
		Egfp	siRNA	Haraguchi et al., 2004 [120]
		Nek2A	dsRNA	Sonn et al., 2004 [123]
		Oct-4	siRNA	Haraguchi et al., 2004 [120]
		Par3	dsRNA	Plusa et al., 2005 [150]
		aPKC	dsRNA	Plusa et al., 2005 [150]
Porcine	Oocyte	Plk1	dsRNA	Anger et al., 2004 131]
	Embryo	Karyopherins a2, a3	dsRNA	Cabot and Prather, 2003 [132]
Bovine	Oocyte	C-mos	dsRNA	Nganvongpanit et al., 2006 [135]
		Cyclin B1	dsRNA	Paradis et al., 2005 [136]
	Embryo	Connexin 43	dsRNA	Tesfaye et al., 2007 [133]
		E-cadherin	dsRNA	Nganvongpanit et al., 2006 [134], Tesfaye et al., 2007 [133]
		Oct-4	dsRNA	Nganvongpanit et al., 2006 [134] ; Nganvongpanit et al., 2006 [135]

for studying the effects of genes and inactivation of genes in livestock for example cattle, swine and poultry in addition to human. Also, dsRNA was used to investigate the possible role of Gdf-9 in mediating oocyte regulation of cumulus expansion [119]. Fully-grown mouse oocytes injected with Gdf-9 dsRNA, Bmp15 dsRNA or injection buffer were cultured for 24 h and processed for measurement of Gdf-9 and Bmp-15 mRNA levels using real-time RT-PCR, and for measurement of Gdf-9 protein levels using western blotting and immunofluorescence staining techniques. Injection with Gdf-9 dsRNA knocked down Gdf-9 but not Bmp-15 mRNA expression in oocytes, and vice versa. Furthermore, Gdf-9 protein levels were reduced in the Gdf-9 dsRNA injected oocytes. To investigate the role of Gdf-9 in cumulus expansion, two endpoints genes namely: Has-2 and Ptgs-2 were used to evaluate cumulus expansion. The mRNA levels were measured in cumulus cells using real-time RT-PCR and assessment of cumulus expansion was undertaken morphologically. After 24 h of culture in the presence of 0.5 U/ml follicle

stimulating hormone (FSH), cumulus shells co-cultured with buffer and Bmp-15 dsRNA injected oocytes exhibited a high degree of expansion, while cumulus shells co-cultured with Gdf-9 dsRNA injected oocytes exhibited only limited expansion. Supporting this observation, after 8 h of co-culture Has-2 and Ptgs-2 mRNA levels were lower in cumulus cells co-cultured with Gdf-9 dsRNA injected oocytes than in those co-cultured with buffer injected oocytes. These results strongly support the concept that Gdf-9 is a key mediator of oocyte-enabled cumulus expansion in mice. In bovine, RNAi represents a useful technique to study gene function in oocyte. The injection of Cyclin B1 dsRNA resulted in a decrease in Cyclin B1 mRNA and protein, while the Cyclin B2 mRNA remained unaffected. Furthermore, the injection of GFP dsRNA did not interfere with Cyclin B1 mRNA or protein with the ability of the oocyte to mature properly [136]. Moreover, the study conducted by our group has shown the E-cadherin transcripts and proteins were reduced after embryos were treated with E-cadherin dsRNA, and the blastocyte rates in those embryos was found to be lower as compared with that of the control group [134]. Moreover, in the other study of our group, microinjection of C-mos dsRNA has resulted in 70% reduction of C-mos transcript after maturation compared to the water injected and uninjected controls ($P < 0.01$). Microinjection of zygotes with Oct-4 dsRNA has resulted in 72% reduction in transcript abundance at the blastocyst stage compared to the uninjected control zygotes ($P < 0.01$). From oocytes injected with C-mos dsRNA 60% showed the extrusion of first polar body compared to 50% in water injected and 44% in uninjected controls. Moreover, only oocytes injected with C-mos dsRNA showed spontaneous activation [135]. Those studies have given evidence that the use of sequence specific dsRNA or siRNA to induce RNAi in mammalian oocytes and embryos to suppress maternal and embryonic transcripts leads to a subsequent reduction in functional protein expression and a distinct developmental phenotype. Furthermore, those results demonstrated that sequence specific dsRNA can be used to knockdown maternal or embryonic transcripts in mammalian embryogenesis and used as a tool to study the function of genes.

5. CONCLUSION

Microarray has become a very important tool for studying gene expression profiles under various conditions. Based on its main function in the identification of transcripts whose abundance differs between samples, microarray has made important contributions to both basic and applied research, and promise to change the practice of several research fields. This technique allows us to study global gene expression in the complex of metabolic pathways. Emerging from their roots in gene screening and target identification microarrays are now being applied to disease characterization and developmental biology. Generally, microarray has come to play a central role in the rapidly evolving field of transcriptomics and the advantage of microarray is fulfilled with the improvement of RNAi technique. In fact, RNAi is a revolution in the field of animal molecular genetics that it has enormous potential for engineering control of gene expression, as well as for the use of a tool in functional genomics. The ability to manipulate RNAi has a wide variety of practical applications of biotechnology ranging from molecular biology to gene therapy. The use of RNAi as a method to alter gene expression in mammalian embryo has been attempted in a diverse rate of success. Analysis of more genes using RNAi in bovine including other

mammalian species will help researchers to better understand what genes are suitable for RNAi targeting and function of those genes. However, various RNAi approaches need to be compared and standard protocols must be developed for a better use. The RNAi transgenic approach is very interesting for studies of early mammalian development.

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