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Expression dynamics of microRNA biogenesis during preimplantation mouse development

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ABSTRACT

The role of microRNAs (miRNAs) in early development, and particularly in the post-transcriptional regulation of maternal mRNAs remains controversial. Hence, we have assessed how miRNA processing is regulated during preimplantation mouse development, from the fully-grown oocyte to the blastocyst, quantifying the expression of genes whose proteins are involved in miRNAs biogenesis and function. The expression of the *Drosha*, *Dgcr8*, *Exportin 5*, *Dicer*, *Ago1*, *Ago2*, *Ago3*, *Ago4* and *Ago5* genes was downregulated from the zygotic cleavage stage, except for the increase of *Ago1*, *Ago3* and *Ago4* expression in the 2-cell embryo, and of *Ago2* in 4- and 8-cell embryos. These findings suggest that the capacity to process miRNAs, by the considered canonical pathway, diminishes after fertilization, primarily reducing miRNA activity in the later stages of preimplantation development. However, by analyzing the different precursor and mature forms of specific miRNAs that are abundantly expressed in the blastocyst, such as *miR-292-3p* and *miR-292-5p*, we identified miRNA-duplexes and/or miRNAs bound to target mRNAs that may serve as potential stockpiles of miRNAs. In response to the demand, such stockpile could directly provide functional and mature miRNAs.

1. Introduction

In mammals, the accumulation of RNAs in the oocyte is essential to ensure proper embryonic development. However, selective gene regulation is a critical process both in the transition from the oocyte to zygote, and in early embryonic differentiation. After fertilization, there is a rapid shift from a maternal to the embryonic developmental program, which involves the selective repression of many maternal mRNAs and the post-transcriptional regulation of transcripts following activation of the embryonic genome [1]. The destruction of oocyte-specific transcripts, representing about 90% of maternal mRNA at 2-cell embryo, has to be replaced with zygotic or early embryo transcripts coding essential cellular proteins [2].

The biological activity of small non-coding RNAs, including hundreds of microRNAs (miRNAs), is clearly established during development and differentiation through the inhibition of translation and specific mRNA destabilization [3–6]. Thus, miRNAs may mediate the post-transcriptional regulation and turnover of maternal mRNAs that accumulate in the oocyte, thereby modulating the progressive activation of zygotic genes and hence, early differentiation from zygote to blastocyst. Such modulatory activity has been demonstrated in some organisms, such as zebrafish [7], although it remains controversial in others, including mice [8–10].

The first step in such regulatory pathways affects the biogenesis of endogenous small RNAs, primarily miRNAs. The canonical pathway from transcription of the miRNA precursor, the pri-miRNA, to the formation of the functional and mature miRNA is initiated by RNA polymerase II. After transcription, the RNase DROSHA, as part of a complex formed with the DGCR8 protein (DiGeorge syndrome critical region gene 8), cleaves the pri-miRNA in the nucleus to generate the pre-miRNA, a 60–70 nt intermediary molecule. The stem–loop structures of the pre-miRNAs are then exported to the cytoplasm by the exportine-5 protein, where the pre-miRNAs are excised by DICER to produce 22–25 nt duplex RNAs from which the RISC (RNA-induced silencing complex) is formed by the incorporation of one RNA strand or mature miRNA. This mature form of the miRNA in turn targets a complementary mRNA, either cleaving it or repressing its translation [11–13]. Finally, the proteins of the Argonaute (AGO) family are essential for RISC activity and they probably also regulate other aspects of miRNA biogenesis and function [14,15].

The abundance of miRNAs in specific cell types, tissues or developmental programs is primarily regulated through pri-miRNA transcription. However, the abundance and function of miRNAs, both during normal and pathological developmental processes, may also be modulated at each of the different steps of miRNA biogenesis from the precursor to the mature forms [16]. That is, the relative expression of genes encoding the key proteins involved in miRNA biogenesis and activity will influence the overall physiological activity of miRNAs in specific cells during development. Since the programs regulating gene expression are very dynamic during mammalian preimplantation development, analyzing expression profiles from the unfertilized oocyte to the zygote, 2-cell, 4-cell, 8-cell,

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morula and blastocyst stages of development may help elucidate the regulatory elements involved in the biogenesis and function of miRNAs and the potential roles of miRNAs in the maternal to zygotic transition.

We previously characterized the expression of genes encoding proteins that participate in the RNA interference pathways mediated by miRNAs and in male mice, we found very different patterns of expression in differentiating germ cells than in the somatic cells in the testis [17]. Although expression patterns of some microRNA pathway genes during preimplantation development, representing partial aspects of the process, have been previously reported, the results are not entirely conclusive [18–22]. To obtain a global overview of the dynamic biogenesis of miRNAs in early embryogenesis, we used RT-qPCR to compare the expression of genes encoding proteins with pivotal roles in such processes and in miRNA activity, namely *Drosha*, *Dgcr8*, *Exportin 5*, *Dicer*, *Ago1*, *Ago2*, *Ago3*, *Ago4* and *Ago5* (officially known as *RNasen*, *Dgcr8*, *Xpo5*, *Dicer1*, *Eif2c1*, *Eif2c2*, *Eif2c3*, *Eif2c4* and *Eif2c5* respectively). Gene expression was quantified in unfertilized fully-grown oocytes, in zygotes and in 2-cell, 4-cell, 8-cell, morula and blastocyst embryos.

To correlate the expression of the elements that regulate miRNA biogenesis with the accumulation of pri-miRNA, pre-miRNA and mature miRNA forms, we analyzed the expression of four miRNAs with distinct and defined patterns of expression during preimplantation development: *mmu-miR-let7a*, *mmu-miR-20a*, *mmu-miR-292-3p* and *mmu-miR-292-5p*. Our data strongly suggest that mature forms of some of these miRNAs, with expression in the later stages of preimplantation development, could also be generated from stockpiles of these molecules by recycling their double-stranded duplex forms and/or those bound to target mRNAs.

2. Materials and methods

2.1. Animals

All procedures relating to the care and handling of animals were carried out in accordance with the regulations of the Consejo Superior de Investigaciones Científicas (CSIC) Bioethics Committee (which specifically approved this study, permit #: PI071007) and the relevant European Commission (EC) guidelines (directive 86/609/EEC). C57BL6 and DBA/6J mice were bred at the CIB-CSIC bioterium under specific pathogen-free (SPF), temperature (22 ± 1 °C) and humidity-controlled (50–55%) conditions, on 12 h light/dark cycles and with *ad libitum* access to food and water.

2.2. Oocyte/embryo collection and culture

Fully-grown oocytes and fertilized eggs were collected from the oviducts of mice as described previously [23]. Briefly, superovulation was induced in 4–5 week old C57BL6 female mice by intraperitoneal injection of 5 IU pregnant mare serum (PMSG), followed 48 h later by 5 IU human chorionic gonadotropin (HCG). Female mice were mated with DBA/6J males after HCG administration. Fertilized and unfertilized eggs were treated with hyaluronidase (300 μg/ml, Sigma H3884) to remove the cumulus cells, and they were washed by passing through several drops of M2 medium (Sigma M7167). Fertilized eggs were cultured *in vitro* to the blastocyst stage in M16 medium (Sigma M7292) in a 5% CO_2 atmosphere at 37 °C. Sets of 10 cells/embryos were collected from each pre-implantation developmental stage (unfertilized oocytes and zygote, two-cell, four-cell, eight-cell, morula and blastocyst embryos) to analyze the genes involved in miRNA biogenesis and mature miRNAs by PCR. For PCR analysis of miRNA precursor genes and mature miRNAs, cohorts of 30 embryos were collected. In all cases, the zona pellucida was removed by incubation in 30 μl of prewarmed acidic Tyrode solution under mineral oil for approximately 15 s, followed by careful washing in three drops of M2 medium under a stereomicroscope. Each set of samples was stored in separate 200 μl Eppendorf tubes at −80 °C in 2 μl of RNase-free PBS until use.

2.3. Analysis of expression of mRNA by RT-qPCR

Reverse transcription (RT) reactions followed by quantitative PCR (qPCR) were performed using whole-oocyte or preimplantation embryo lysates in the presence of NP-40 [24]. After heat-shock treatment at 95 °C for 5 min, RNA was specifically retrotranscribed after adding to the lysate 10 μl of a mix containing 2.5 μM Oligo dT17, 1× First-Strand Buffer (Invitrogen), 0.01 M dithiothreitol (DTT), 2 U of RNase inhibitor (RNAsin Promega), 0.5 mM of each dNTP and two hundred units of superscript II (Invitrogen). The reaction was made up to a final volume of 20 μl with RNase-free water.

RNA from testis and brain was isolated using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. RNA was dissolved in RNase free water and the RNA concentrations were verified by measuring the absorbance (A260/280 ratio) on a NanoDrop Spectrophotometer ND-1000 (NanoDrop). Samples were stored at −80 °C until 0.5 μg of total RNA was used to synthesize cDNA in reactions with 2.5 μM Oligo dT17, 1× First-Strand Buffer (Invitrogen), 0.01 M dithiothreitol (DTT), 2 U of RNase inhibitor (RNAsin Promega), 0.5 mM of each dNTP and two hundred units of superscript II (Invitrogen). The reaction was made up to a final volume of 20 μl with RNase-free water.

The cDNAs resulting from the reverse transcription reaction were amplified by real-time quantitative PCR. Reactions were performed by adding 10 μl of 2× SYBR Green PCR supermix (Bio-Rad) to each well containing 4 μl of template and 0.0625 μM of each specific primer in a 20 μl reaction volume. PCR profiles were obtained using the iQ5 Detection System (Bio-Rad) as follows: denaturation at 95 °C for 10 min; 50 cycles of amplification of 15 s at 95 °C, 30 s at 61.4 °C and 1 min at 72 °C. At the end of each reaction the cycle threshold (Ct) was manually set at the level that reflected the best kinetic PCR parameters, and melting curves were acquired and analyzed.

2.4. TaqMan low density arrays

A pool of oocytes and preimplantational embryos was analyzed using TaqMan® rodent microRNA arrays v2.0 (Applied Biosystems). These arrays represent 313 unique assays, specific to mouse and rat mature miRNAs, derived from Sanger miRBase v10. RT-qPCR reactions were carried out using the manufacturer's recommendation. Briefly, 100 oocytes or 100 embryos for each preimplantation stage, were treated with NP-40 0.1% in RT mixture buffer. Cell lysates were used to reverse transcription of RNA by Megaplex RT Primers and TaqMan miRNA reverse transcription kit (Applied Biosystems). Quantitative RT-qPCR was performed using TaqMan universal PCR master mix using the following conditions: 10 min at 94.5 °C followed by 40 cycles of 97 °C for 30 s and 59.7 °C for 1 min in the Applied Biosystems 7900 HT thermal cycler. Data was normalized using the $2^{\Delta\Delta Ct}$ method and *U6* to normalize gene expression.

2.5. Amplification of precursor and mature miRNAs

Cells were lysed as indicated for mRNA. The primers to analyze the miRNA precursor molecules were designed as described previously [25,26]. To amplify pri- and pre-microRNAs the same reverse primer was used. The forward primers designed in the stem portion of the precursor molecules allowed the amplification of each pre-microRNA + pri-miRNA. To amplify each specific pri-miRNA, the forward primers were designed to anneal ~50 nt 5′ of the corresponding pre-microRNA (Fig. 3D). The amount of pre-miRNA was calculated using the equation [26]:

$$\text{pre} - \text{miRNA} = 2^{-Ct(\text{pre}-\text{microRNA}+\text{pri}-\text{miRNA})} - 2^{-\text{Ct}(\text{pre}-\text{microRNA})}.$$

Commercial RT-primer and PCR-primer-TaqMan® were used to analyze the mature miRNA forms (Applied Biosystems). The standard