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Profiling Maternal mRNA Translation During Oocyte Development

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Abstract

With the progress in our understanding of germ cell development, there is an emerging need to investigate the mechanisms of mRNA translation functioning in these cells. Indeed, posttranscriptional regulations of gene expression drive the most important transitions of the germ cell life cycle. Here we describe a strategy to measure mRNA translation in the oocyte, taking advantage of an approach originally developed to identify the transcriptome of a subgroup of cells in a complex cell mixture. This technique takes advantage of the "RiboTag" approach to express an HA-tag on the large ribosomal subunit of the ribosomes in the oocyte. Immunoprecipitation of the extracts followed by qPCR or RNAseq is used to identify mRNAs actively translated.

Keywords

RNA; Ribosomes; Translation; Oocyte; Meiosis

1 Introduction

In numerous biological settings, the analysis of cell transcriptomics does not provide sufficient insight into the stage of differentiation of a cell, nor of its functional properties. This is in part due to the many layers of regulations involved in posttranscriptional control of gene expression. Conversely, investigation into the pattern of translation of expressed mRNAs provides a more accurate picture of protein synthesis in different developmental or functional states. This is particularly true for germ cells, including the female gamete, or oocyte. Germ cells often use translational rather than transcriptional regulation to control their most critical developmental transitions throughout their life cycle. This dependence on translation is even more striking for the fully grown oocyte. Transcription is mostly quiescent in these cells and gene expression is regulated by translational rather than transcriptional mechanisms. In the mouse and human gametes, these posttranscriptional regulations support embryo development until the zygotic genome is activated in the preimplantation embryo. Maternal mRNA translational regulation drives development even

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further up to the pregastrula stage in model organisms such as *Drosophila* and *Xenopus*. Thus, genome reprogramming and the meiosis/mitosis switch of the cell cycle in the zygote are controlled predominantly at the translational level.

Recently, several genome-wide strategies have been devised to accurately measure mRNA translation in a cell, including ribosome profiling and genome-wide polyadenylation [1, 2]. However, the scarcity of oocytes present in the gonad has been a major challenge to the routine application of these techniques. Translation measurements by polysome arrays have been used for oocytes [3]; however the large number of oocytes required has limited the measurements to one or two stages of oocyte maturation. Here, we describe an adaptation of a strategy developed to probe transcription in subpopulations of cells within a complex tissue to measure translation in the oocyte with a high temporal resolution. This technique was first described as a tool for transcriptome analysis in knock-in mice expressing Cre recombinase under the control of the dopamine transport (DAT, *Slc6a3*) promoter, transgenic mice expressing Cre under the control of dopamine, cAMP-regulated phosphoprotein (DARPP32, *Ppp1r1b*) and antimullerian hormone (*Amh*) promoters [4]. This RiboTag technique was then applied to study mRNA recruitment in Sertoli and Leydig cells in vivo upon hormonal stimulation, demonstrating the role of LH stimulation on the translational machinery in Leydig cells [5]. Several other studies have made use of the RiboTag technique to study mRNA recruitment and transcriptome analyses in specific cell populations within complex tissues upon different hormonal or pharmacological inputs. We demonstrated that the RiboTag is a powerful technique to explore protein synthesis through mRNA recruitment responsible for driving the oocyte meiotic cell cycle [6]. It has been useful also to characterize the role of the different RNA binding proteins in that process. Several other reports have since been published using RiboTag IP coupled to RNAseq to mine and validate different target proteins fundamental in oocyte maturation [7].

The RiboTag approach takes advantage of a genetic strategy to tag a ribosomal protein and express it in a cell specific manner. To tag ribosomes exclusively in the oocytes, a genetically engineered mouse carrying a floxed construct targeting the last exon of RPL 22, a protein of the large ribosomal subunit, is mated to a transgenic mouse that expresses the Cre recombinase exclusively in the oocyte (Fig. 1a). The ZP3-Cre mouse has been extensively used for this purpose [8]. Female mice derived from this mating, once checked for the correct genotype, are used for the experiments. This approach is useful to measure translation of a specific mRNA and for genome-wide analysis of translation (Fig. 1b).

2 Materials

Prepare stock solutions (*see* Note 1) and aliquot them into amounts sufficient for single use. Store stock solutions and materials in an “RNase-free” area (*see* Note 2). Prepare all the

1. This is a 3-day procedure (not including sample collection):

   a. Day 1—Prepare all working solutions, prepare the RNase- free station, label the tubes and confirm that all the equipment is reserved and available specifically for this experiment.

   b. Day 2—Lyse the samples and set up RNA-IP (incubate o/n).

   c. Day 3—Wash RNA-IP, elute and extract RNA.

"2×" buffers on the day before the actual experiment. The present protocol was optimized to specific equipment and products routinely used in the laboratory. Different brands or products might require further optimization. We used the RNeasy Micro Plus Kit for RNA extraction.

2.1 Oocyte Collection and Mouse Genotype

The RiboTag technique is based on the transgenic mouse model B6.129-Rpl22tm1.1Psam/J (Ribotag), with a targeted mutation that allows conditional expression of the ribosomal protein L22 (Rpl22 tagged with three copies of the hemagglutinin (HA) epitope, developed by Paul S Amieux and colleagues [4], which is available for purchase from the Jackson Laboratories. A specific Cre line should be selected according to the developmental stage of interest. For oocyte maturation females from the C57BL/6-TgN(Zp3- cre)82Knw transgenic line, where the Cre expression is controlled by the regulatory sequences from the mouse *zona pellucida* 3 (Zp3cre) gene, were crossed to B6.129-Rpl22tm1.1Psam/J homozygous males to produce C57BL/6-Zp3/cre- Rpl22tm1.1Psam (Zp3/cre-Ribotag) mice. Zp3/cre-Ribotag 21–24 day old females were stimulated with 5U of PMSG and oocytes collected after 48 h.

2× Homogenization Buffer (2× HB): 100 mM Tris–HCl, pH 7.4, 200 mM KCl, 24 mM $MgCl_2$, 2% NP40, RNase-free water.

Complete HB (cHB): 2× HB, Protease inhibitors, 1 mM dithiothreitol (DTT), RNAse inhibitors (RNAseOUT or equivalent), 100 μg/mL cycloheximide, 1 mg/mL heparin, RNase-free water.

2× Wash Buffer (2× WB): 100 mM Tris–HCl, pH 7.4, 600 mM KCl, 24 mM $MgCl_2$, 2% NP40, RNase-free water.

Complete Wash Buffer + Urea (cWBU): 2× WB, 1 mM DTT,

RNAse inhibitors, 100 μg/mL cycloheximide, RNAse-free water, 1 M urea.

Complete WB (cWB): 2× WB, 1 mM DTT, RNAse inhibitors, 100 μg/mL cycloheximide, RNAse-free water.

3 Methods

3.1 Oocyte Collection

1. Wash and collect oocytes from mice of the correct genotype in PBS with 1% polyvinylpyrrolidone (PBS-PVP).
2. Flash-freeze in liquid nitrogen.
3. Store in a minimal volume at −80 ° C (*see* Notes 3 and 4).

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2. Have all the equipment ready, cleaned, and RNase free; it is important that during the actual procedure there is as minimal interference. Avoid using centrifuges and rotors routinely used for non-RNA work.
3. If pooling different oocyte preparations, it is important to always collect them in the least amount of PBS possible.

3.1.1 Day 1

1. Prepare 2× HB and 2× WB.
2. Ready the RNA extraction and purification columns as directed by the kit.
3. Label all the tubes necessary for the entire procedure and store in an RNase-free environment.

3.1.2 Day 2

1. Prepare HB and cHB.

3.2 Prepare Dynabeads Protein G

1. Wash magnetic beads with PBS[1].
2. Add 30 μL (per sample) of magnetic beads to 250 μL HB[2].
3. Incubate on rotor for 5 min at 4 °C.
4. Repeat two times.
5. Wash with cHB.
6. Complete total volume up to 250 μL with cHB.
7. Incubate on rotor for 5 min at 4 °C.
8. Repeat the process two more times.
9. After the final wash, magnetic beads should be eluted in cHB to allow for 20 μL per sample, plus the extra volume required to preclear the samples and to compensate for pipetting errors.

3.3 Prepare Lysate

1. Collect oocytes[3] in a single tube (5–10 μL PBS/PVP), add 250 μL cHB and vortex for 15 s (*see* Note 5).
2. Lyse oocytes through freezing–thawing with liquid nitrogen, leave on ice for 10 min.
3. Spin down at 11,000 × *g* for 15 min at 4 °C.
4. Save supernatant for RNA-IP.

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4. Collect enough material to allow for an RNA-IP with the antibody of interest and the respective negative control with IgG. As an example, one should plan to use extracts from at least 70 oocytes for the HA IP and 70 oocytes for the nonimmune IgG.

[1] The volume of slurry will vary according to the number of samples planned. It is important to remember that each sample needs to be divided into 2 (HA antibody and control IgG).

[2] Always add an excess of 20–30 μL of magnetic beads to preclear the sample and another 20–30 μL to compensate for pipetting errors.

[3] The number of oocytes can vary; in our past experience in the lab, we were able to collect sufficient RNA with a minimum of 75 oocytes per RNA-IP (HA antibody and IgG).

5. When performing detailed time courses of ribosome loading onto the mRNA of interest, it is not always possible to collect all necessary material in one session. In this case, it is important to always prepare pools of oocytes for all time points during any given collection session. The aim is to build a pool of oocytes collected on different days from different mice for each time point so that the biological variable is uniformly distributed throughout all the samples.

3.4 Preclear Protein Lysate

1. Add 20 μL of washed beads to the protein lysate.
2. Incubate on rotor for 30 min at 4 °C.
3. Place the sample tube in the magnetic rack to allow for separation of the beads from the preclear step.
4. Collect supernatant for RNA-IP.

3.5 RiboTag IP

1. Collect 10% of the precleared lysate for “Input RNA” measurements.
2. Aliquot the precleared lysate equally between the HA antibody tube and IgG control.
3. Add 2 μg of antibody to each sample (*see* Note 6).
4. Make up volume to 300 μL with cHB.
5. Incubate on rotor for 2 h at 4C.
6. Add 30 μL of washed magnetic beads.
7. Incubate on rotor o/n at 4 °C.

3.5.1 Day 3

1. Wash pelleted beads with 0.75 mL of cWB for 10 min in the rotor.
2. Repeat washes five times (three times with cWB + 2 times with cWBU, *see* Notes 7 and 8).
3. Add 250 μl of supplemented RLT (provided by the RNeasy micro Plus Kit).
4. Vortex for 30 s.

3.6 RNA Extraction (RNeasy MicroPlus Kit)

1. Following the manufacturer instructions, elute in 9 μL dH_2O (*see* Notes 9 and 10)[4]

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6. The amount of antibody used needs to be properly adjusted so that it does not exceed the capacity of the beads. Bead capacity is provided by the suppliers. One can test whether the capture is complete by performing a Western blot of the supernatant after IP with antibodies against IgG.

7. The stringency of the wash of the bead/ribosome/mRNA pellet can be adjusted by increasing the number of washes with 1 M urea. Performing all the washes in 1 M Urea decreases the nonspecific IgG signal of mRNA measured by qPCR. However, it may also decrease the signal in the HA-AB specific pellet. The most suitable conditions for the mRNA of interest can be established by testing the recovery in the IP pellet after different washes with urea.

8. In line with that mentioned above, it should be considered that quantification of the mRNA recovered in the RiboTag IP pellet is a relative measurement as it is the total amount RNA recovered minus the amount recovered in the nonspecific IP (IgG IP). Thus, the strategy is used to detect differences between two conditions but it does not provide absolute measurements of mRNA/ribosome interactions, unless correction for the recovery is introduced. An estimate of the amount of RNA recovered in the pellet (i.e., percentage of any given mRNA bound to ribosomes) can be obtained by copy number qPCR measurements. This can be obtained by running qPCRs with a standard curve for the specific mRNA of interest and by converting the Ct number to copy number by using the following equation: Number of copies = (× ng * 6.0221 × 10^{23} mole cules/mole)/[(N*660 g/mole) * 1 × 10^{9} ng/g)].

9. In the past experience of the laboratory, we determined that 70 oocytes per IP is a minimum number of oocytes that can be used. We have obtained inconsistent data by decreasing the number of oocytes further because of the decrease in recovery with diluted RNA

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solution. Although not tested in the lab, one could attempt to increase the recovery of the IP by adding carrier nucleic acid to minimize losses with extremely diluted concentrations of cell extracts.

[10]For a genome-wide analysis of mRNA translation, the RiboTag IP is followed by preparation of libraries for RNAseq. In our experience, we have successfully prepared useful libraries by HA immunoprecipitation of the equivalent of 150–200 oocytes. The libraries yield is sufficient for RNAseq with a depth of reads of about 50 M reads. It is useful to save an aliquot of the oocyte extracts before immunoprecipitation. This sample can be used to prepare a library and used as an "input" reference during the analysis of the data.

[4]This volume was determined because we use all the RNA for the cDNA synthesis reaction using the Reverse-Transcription SupIII Kit from Invitrogen, which uses 8 μL of RNA per reaction.

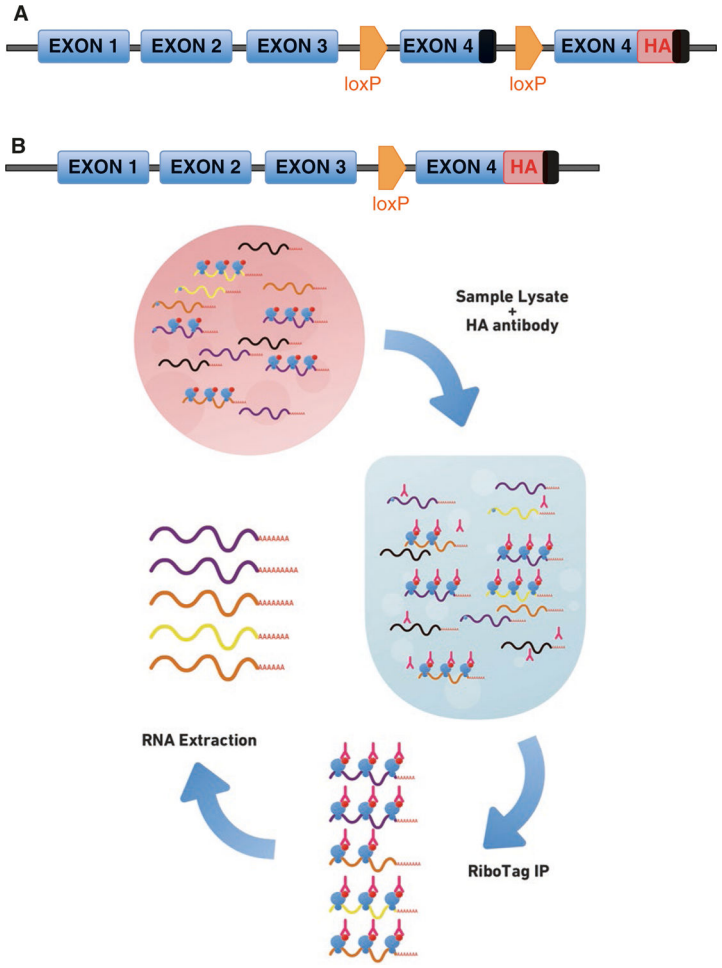

Fig. 1.

(**a**) RiboTag genomic locus strategy. Schematic representation of the RiboTag targeting strategy of the genomic locus of the Rpl22. Two loxP sites were inserted 5′ and 3′ to the wild-type exon 4 of *Rpl22,* together with a modified *Rpl22* exon 4 containing the HA epitope tag before the stop codon at the 3′ of the wild-type exon 4 (**a**). Crossing the resulting mouse with a Cre recombinase-expressing mouse deletes the wild-type exon 4 in the targeted cell, replacing it with an Rpl22-HA exon 4 (**b**). Blue boxes represent the different exons of Rpl22, orange arrows represent the loxP sites, HA red squares denotes the HA tag epitope and black boxes represent the stop codon. Adapted from [4]. (**b**) Schematic representation of the RiboTag strategy. Cre-RiboTag oocytes expressing multiple mRNAs and ribosomes tagged with the HA epitope are collected at specific points of development. Oocytes are lyzed and an HA antibody is added to this sample (after initial preclear step). Incubation overnight allows for the selection of mRNAs bound by HA-tagged ribosomes by magnetic immunoprecipitation. RNA extraction is accomplished by releasing the mRNA from the ribosome–antibody complexes, and these samples are stored for further analysis (qPCR or mRNA libraries)