Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1779 (2008) 306–311
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbagrm

# The expression patterns of genes involved in the RNAi pathways are tissue-dependent and differ in the germ and somatic cells of mouse testis

Emilio González-González, Pedro P. López-Casas, Jesús del Mazo *

*Department of Cell and Developmental Biology, Centro de Investigaciones Biológicas, (CSIC), Ramiro de Maeztu 9, E-28040 Madrid, Spain*

Received 6 November 2007; received in revised form 14 January 2008; accepted 22 January 2008
Available online 14 February 2008

**Abstract**

Different RNA interference (RNAi) components participate in post-transcriptional regulation via RNA silencing. The expression pattern of the genes *Drosha* and *Dicer* and the members of the Argonaute family *Ago1*, *Ago2*, *Ago3* and *Ago4*, all elements participating in the RNAi pathways, were investigated in mouse somatic tissues and testis using quantitative RT-PCR. Expression patterns of different testis cells and those emerging during testis development were also investigated. The differential patterns of expression seen suggest potential pleiotropic roles for certain components of the RNAi machinery. Both spermatocytes and spermatids showed a defined gene expression pattern. The strong expression of Ago4 in germ cells suggests that this protein plays a key role in germ-cell differentiation in the seminiferous epithelium.


*Keywords:* RNAi; Drosha; Dicer; Argonaute; Spermatogenesis; Germ cell; Sertoli cell

## 1. Introduction

RNA interference (RNAi) is mediated by small interfering RNAs (siRNAs) and microRNAs (miRNAs) [1]. In differentiation and development, RNAi operates as a regulator of gene expression [2]. Such regulation is probably more important in complex processes — certainly the gene expression that governs the events in male germ-cell differentiation requires very precise control [3,4]. RNAi mechanisms may act throughout spermatogenesis [5,6]; in support of this, Yu et al. [7] report several miRNAs to be differentially expressed during the development of the seminiferous epithelium.

Although the intricate process of gene regulation by RNAi is not fully understood, many conserved elements participating in the RNAi pathway have been identified [8]. This pathway involves two main steps: (1) the processing of endogenous miRNA or dsRNA by the RNaseIII enzymes Drosha and Dicer, and (2) the cleavage of target RNA via the RNA-induced silencing complex (RISC) (see the recent review by Hammond 2005) [9].

Argonaute proteins – components of RISC – are highly basic proteins [10] characterized by two domains: the PAZ domain (which interacts with the 3' end of siRNAs) and the PIWI domain (which binds to the 5' end of siRNAs as well as the target RNA to be silenced). The Argonaute family can be subdivided into the Ago and Piwi subfamilies. The Ago members are generally considered to be ubiquitously expressed and to be involved in siRNA and miRNA function [11]. The expression of the Piwi members is more restricted to germ-line cells [12]. This is based on the isolation, from mouse testis, of a new class of small RNAs known as piwi-interacting RNAs (piRNAs) [13–16]. AGO1 and AGO2 form complexes with Dicer. AGO2 alone, however, is responsible for the cleavage activity of RISC, presumably through the RNAse H like structure in the PIWI domain [17]. AGO1, AGO2, AGO3 and AGO4 have been identified in mRNA decay centers, or processing bodies (p-bodies) as they are known in somatic cells. It has been suggested that AGO proteins act through mRNA degradation or via the inhibition of translation [18]. Recently, Kotaja et al. [19] localized Dicer, AGO2, AGO3 and Miwi (a murine Piwi-family member) along with miRNAs in germ-cell specific cytoplasmic chromatoid bodies (c-bodies). These structures are similar to p-bodies and are thought to be involved in the storage and processing of haploid cell transcripts.

* Corresponding author. Tel.: +34 91 8373112, 4324; fax: +34 91 5360432.
*E-mail address:* jdelmazo@cib.csic.es (J. del Mazo).

1874-9399/$ - see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbagrm.2008.01.007

Table 1
Primers used for RT-PCR

| Gene | Primer sequences Forward/reverse | Accession No. |
|---|---|---|
| *Drosha* | 5'ggaccatcacgaaggacacttg3'<br>5'atgcccagttcctctgctacct3 | NM_026799 |
| *Dicer* | 5'ggatgcgatgtgctatctgga3'<br>5'gcactgctccgtgtgcaa3' | NM_148948 |
| *Ago1* | 5'gaagacgccagtgtatgctgaa3'<br>5'atcttgaggcagaggttggaca3' | AB081471 |
| *Ago2* | 5'gccgtccttcccactaccac3'<br>5'ggtattgacacagagcgtgtgc3 | AB081472 |
| *Ago3* | 5'ttggaagaagcggcaacatc3'<br>5'gatagtgtgaaggacggctggt3' | AB081473 |
| *Ago4* | 5'acacgctccgtctccattcc3'<br>5'gactgccttccgcactgtca3' | AB081474 |
| *Rps16* | 5'ttctgggcaaggagcgatt3'<br>5'gatggactgtcggatggca3' | BC082286 |

In mammalian testis, different types of small RNAs may operate as modulators of gene expression. However, the participation of RNAi machinery elements in specific cell differentiation programs such as spermatogenesis has not been established. To investigate the participation of RNAi machinery elements in spermatogenesis, the expression of the genes that encode Drosha, Dicer, AGO1, AGO2, AGO3 and AGO4 (officially known as *Rnasen*, *Dicer1*, *Eif2c1*, *Eif2c2*, *Eif2c3* and *Eif2c4*) was analyzed (using quantitative RT-PCR [qRT-PCR]) in the developing testis, in different seminiferous epithelium types, in a number of somatic tissues, and in different seminiferous epithelium cells types.

## 2. Methods and materials

### 2.1. Tissue collection and cell isolation

Animals were treated according to the guidelines of the CSIC Bioethics Committee. A CD-1 mouse colony was maintained in a temperature- and humidity-controlled room. Food and water were provided *ad libitum*. Somatic tissues (brain, spleen, heart, muscle and lung) were obtained from adult mice. Whole testes were obtained from mice at days 6, 10, 14, 18 and 22 postpartum (dpp), and from adults.

Primary Sertoli cells were cultured from the testes of mice aged 16–18 dpp. All cultures were maintained for 2 weeks at 37 °C in a 5% $CO_2$/95% air atmosphere and in Dulbecco's modified Eagle's medium:Ham-F12 medium (Gibco, BRL) (1:1), following standard procedures [20]. Pachytene spermatocytes, round spermatids and elongating spermatids from adult mice were enriched using BSA density gradients on STA-PUT [21]. The purity of all isolated and cultured cells was 90–95%, as determined by morphological criteria and RT-PCR using cell-type specific primers as previously described [22]. Mouse NIH-3T3 fibroblasts were cultured in a 5% $CO_2$/95% air atmosphere at 37 °C in DMEM supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin.

### 2.2. Total RNA extraction and analysis of mRNAs by qRT-PCR

Total RNA from testicular and somatic tissues and from isolated testicular cell types was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Gene expression levels were determined by real time RT-PCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Residual genomic DNA was removed from total RNA by treatment with RQ1 RNase-free DNase (Promega). RNA was reverse transcribed using a 17-mer oligo-dT primer and SuperScript II Reverse Transcriptase (Life Technologies). PCR was performed in a final volume of 25 μl using SYBR Green PCR Master Mix (Applied Biosystem) and 100 nM of each primer. Primer combinations for the specific amplification of the analyzed genes were designed using Primer Express software (version 1.5, Applied Biosystem), following the method of Livak [23]. Table 1 lists the primers used in PCR.

The PCR reaction conditions were as follows: 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min, and a final extension step at 72 °C for 1 min. The PCR products were also examined by melting curve analysis and agarose gel electrophoresis. A replicate was run for each sample omitting the reverse transcription step as a template-negative control. Each experiment was repeated at least three times. The amount of ribosomal protein S16 (*Rps16*) mRNA [24] was measured in each sample for normalization purposes. Gene expression data during seminiferous epithelium development were presented using a modification of the $2^{-\Delta\Delta Ct}$ method [25,26]. Expression in adult testis was used as the calibrator. The data for tissues and cell types normalized only to *S16*, without the use of a calibrator are presented.

### 2.3. Statistical analysis

Since the range of gene expression values calculated with the $2^{-\Delta\Delta Ct}$ method is asymmetrically distributed [26], the values recorded were log-transformed to generate a normal distribution. The Student's *t* test was then used to compare the mean expression of each gene for each somatic tissue with mean expressions for the testis ($p<0.05$). Pearson correlation coefficients ($r$) were calculated to determine the relationship between the expressions of paired genes in the different

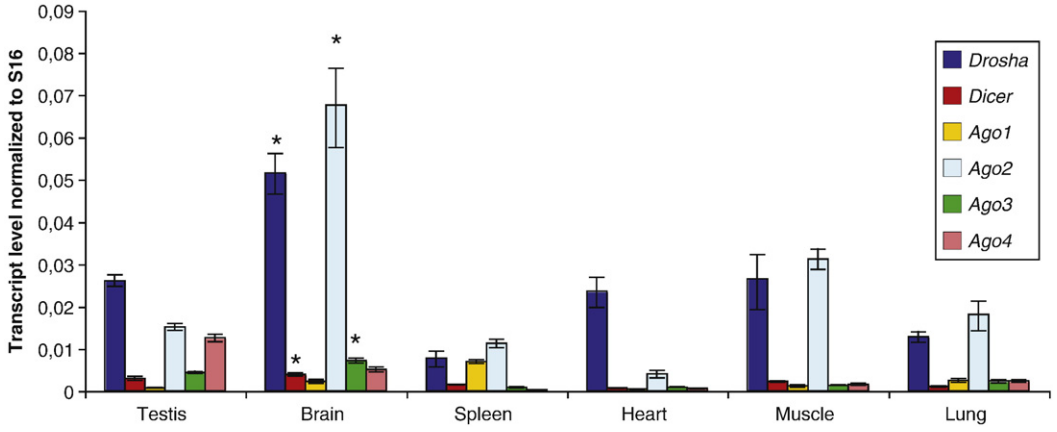


Fig. 1. Quantitative RT-PCR analyses of *Drosha*, *Dicer*, *Ago1*, *Ago2*, *Ago3* and *Ago4* transcripts in different tissues. Error bars represent standard deviations. Asterisks indicate statistical significance.