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Expression Profiles of Long and Short RNAs in the Cytoplasm and Nuclei of Growing Chicken (*Gallus gallus domesticus*) Oocytes

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Abstract—Maternal RNAs accumulated during oocyte maturation are required not only for zygote formation but also for supporting the first embryonic cell divisions until the activation of the embryo genome. The key stages of transcriptome analysis include the adaptation of RNA extraction procedures and characterization of the RNA expression profile. The ovaries of domestic birds represent an adequate model for the exploration of RNA accumulation during oogenesis. In the present study, we optimized the methodologies for RNA extraction from the oocyte cytoplasm and nucleoplasm of egg-laying chickens (*Gallus gallus domesticus*), and characterized the changes in the expression profiles of long and short RNAs during oocyte growth. The cytoplasmic RNA fractions contained 28S and 18S ribosomal RNAs (rRNAs), small RNAs, and long RNAs that were heterogeneous in size. The profiles of the total RNAs from growing oocyte nuclei were dominated by low molecular weight RNAs corresponding in size to transport RNAs, small nuclear RNAs, and short regulatory RNAs. Importantly, nuclei from chicken growing oocytes demonstrated trace amounts or the absence of 28S and 18S rRNAs, which was possibly due to the inactivation of the single nucleolus organizer. We identified three groups of short RNAs differing in size (from 20 to 40 nucleotides) in the chicken oocytes. These may correspond to short regulatory RNA classes: microRNA, siRNA and piRNA. Furthermore, we demonstrated that short RNAs accumulate in the cytoplasm during oocyte growth. We propose that the short RNAs accumulated in the avian oocyte cytoplasm are involved in the regulation of genome functions during the early embryogenesis stages.

Keywords: domestic chicken; noncoding RNAs; short RNAs; maternal RNAs; oocyte; oogenesis; ribosomal RNAs
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Investigations of the spectrum of the transcribed DNA sequences at different tissues of vertebrates, including the most common model organisms, are far from completion. Especially, little attention is paid to the high-throughput analysis of the oocyte transcriptome and the RNAs transferred to embryos from the oocyte cytoplasm (maternal RNA) (Fair et al., 2007; Huang and Wells, 2010). The high throughput analysis, which not only allows to investigate the known transcripts but also to discover new RNAs, involves massive parallel sequencing of the entire set of RNA molecules synthesized in a given cell type or tissue. In particular, investigations of the oocyte transcriptome can reveal the role of the maternal RNA transferred with the oocyte cytoplasm for the early stages of embryo development.

Oocytes of birds represent a convenient model system for the studies of the dynamics of the accumulation of maternal RNA. Ovaries of egg-laying chicken (*Gallus gallus domesticus*) consist of nonhierarchical follicles and maturing follicles, which are classified according to their size (from stage F6 to stage F1). Fetal chicken genome is activated when an embryo contains 30–50 cells 24 h after fertilization (Zagris et al., 1998). Thus, the maternal RNA accumulated in the chicken oocyte during growth is necessary not only for the fertilization and formation of the zygote but also to maintain a certain number of embryonic cell divisions prior to the activation of the embryonic genome.

The oocyte transcriptome can be investigated on the growing oocytes of domestic chicken. The choice of this object is favored by several factors, including the completion of chicken genome sequencing project, average size of chicken genome of 1.2 Gbp, its relatively low repetitive DNA content, the existence of detailed genetic maps of the chicken chromosomes, and the availability of a variety of molecular genetic tools (such as expression microarrays, databases for poly (A)-RNA and short RNA from different tissues and stages of development) (Froman et al., 2006; Cogburn et al., 2007; Nie et al., 2010).

In this manuscript, we present the optimized methods of RNA extraction from the chicken oocyte cytoplasm and nucleoplasm, and characterize the changes in the expression profiles of long heterogeneous and short RNA during oocyte growth.

MATERIALS AND METHODS

Procedure for Preparing Fractions of Oocyte Nuclei and Cytoplasm

We used the standard protocol (http://projects.exeter.ac.uk/lampbrush/protocols.htm) to obtain fractions of the cytoplasm and nucleoplasm from the oocytes of egg-laying females of domestic chickens (*G. g. domesticus*). Nuclei (germinal vesicles) were separated from the rest of the oocyte content and its membranes microsurgically using tungsten needles in the "5 : 1 + phosphates" medium (83.0 mM KCl, 17.0 mM NaCl, 6.5 mM Na_2HPO_4, 3.5 mM KH_2PO_4, 1 mM $MgCl_2$); procedures were monitored under a stereomicroscope (Krasikova et al., 2012). The most optimal material for nuclei isolation appeared to be white nonhierarchical follicles. Microsurgically isolated nuclei, and enucleated (lacking nuclei) oocytes (i.e., oocyte cytoplasm) separated from the layer of follicular cells, were immediately placed on ice and used for RNA isolation.

Isolation of Total RNA from the Cytoplasm and Nucleoplasm in Chicken Oocytes

We optimized the methodology for extracting RNA from various tissues of domestic chicken, including the liver and ovary, as well as from the isolated oocyte nuclei, and the enucleated oocytes of different size. Total RNA was isolated using the TRIzol reagent (Invitrogen) following the recommendations of the manufacturer with some modifications. Namely, after lysis in TRIzol, the extracts were subject to an additional centrifugation at 12000 rev/min for 10 min to get rid of the insoluble fraction. The RNA was precipitated in isopropanol for 20 min at –20°C. For the precipitation of low-molecular weight RNA fractions, the centrifugation time was increased to 30 min. In addition, during the isopropanol precipitation of small amounts of RNA and short RNA, we used RNase-free glycogen at a concentration of 10 μg/mL as a carrier. The RNA pellet was carefully washed (2x) in chilled 75% ethanol with the vortexing pellet during the first wash. The RNA samples were stored in ethanol at –80°C.

Measurement of RNA concentration, Evaluation of the Presence of Impurities, and Analysis of the Total RNA Profile

The concentration of total RNA, as well as the A260 : A280 and A260 : A230 ratios, was determined with a spectrophotometer NanoDrop 2000 (Thermo Scientific, USA). The A260 : A280 ratios in the RNA samples from the cytoplasm of chicken oocytes was not less than 1.9 indicating the absence of the protein contamination of the samples. The A260 : A230 ratio can also be used to evaluate the sample contamination, and its value was above 1.0 for all the RNA samples obtained from the cytoplasmic fraction of the chicken oocytes. The RNA profiles, as well as the degree of sample degradation and the absence of DNA contaminant, was monitored by electrophoresis in a microfluidic chip using Bioanalyzer 2100 (Agilent, USA). The degree of RNA degradation was evaluated based on the index of RNA integrity (RNA Integrity Number, RIN) and the ratio of 28S: 18S rRNAs.

Chromatogram profiling showed a negligible presence of genomic DNA in the chicken cytoplasmic and nuclear RNA samples. Therefore, additional sample purification to eliminate contamination by genomic DNA was not performed.

Analysis of Low Molecular Weight RNAs Profile

The RNA samples were separated in a 10% PAA-gel using a vertical electrophoresis unit, and stained with ethidium bromide. The dye bound to the nucleic acids was visualized under UV light and detected using the gel detection system GDS-800 (UVP, USA). The resulting electrophoreograms were presented using the GellQuantNET program (http://biochemlablabsolutions.com/GelQuantNET.html).

RESULTS AND DISCUSSION

Amount of Total RNAs in Chicken Oocytes

The total RNAs were isolated from various tissues and oocytes of *G. g. domesticus* using a modified protocol allowing us to obtain preparations containing both high and low molecular weight transcripts. The use of glycogen as a carrier significantly increased the amount of RNAs precipitated from the oocyte cytoplasm. The implementation of a modified protocol allowed us to obtain about 1 μg of the total RNA per oocyte, and about 1 ng of total RNA per each isolated nucleus (averaged values), which gives a ratio of 1000 : 1. In comparison to these values, the reported amounts of total RNA obtained by the standard RNA extraction procedure from the Xenopus oocytes (*Xenopus tropicalis*) are slightly higher: about 1 μg of RNA per oocyte and 2 ng of RNA per isolated nucleus (ratio 500 : 1) (Gardner et al., 2012; Simeoni et al., 2012). This is due to the smaller size of the oocytes at the stage of the highest transcriptional activity in the ovaries of domestic chicken.

Profile of High Molecular Weight RNAs in Chicken Oocytes

We compared the profiles of the total RNA from the cytoplasm and nuclei of growing oocytes of *G. g. domesticus* using a microfluidic electrophoresis chip (Fig. 1). We detected the peaks of ribosomal RNAs, small RNAs, and various transcripts heterogeneous in size in the oocyte cytoplasm (Fig. 1b). The presence of 28S and 18S rRNAs in the oocyte cytoplasm allowed us to estimate the degree of RNA degradation in the samples. For cytoplasmic RNA preparations, RIN

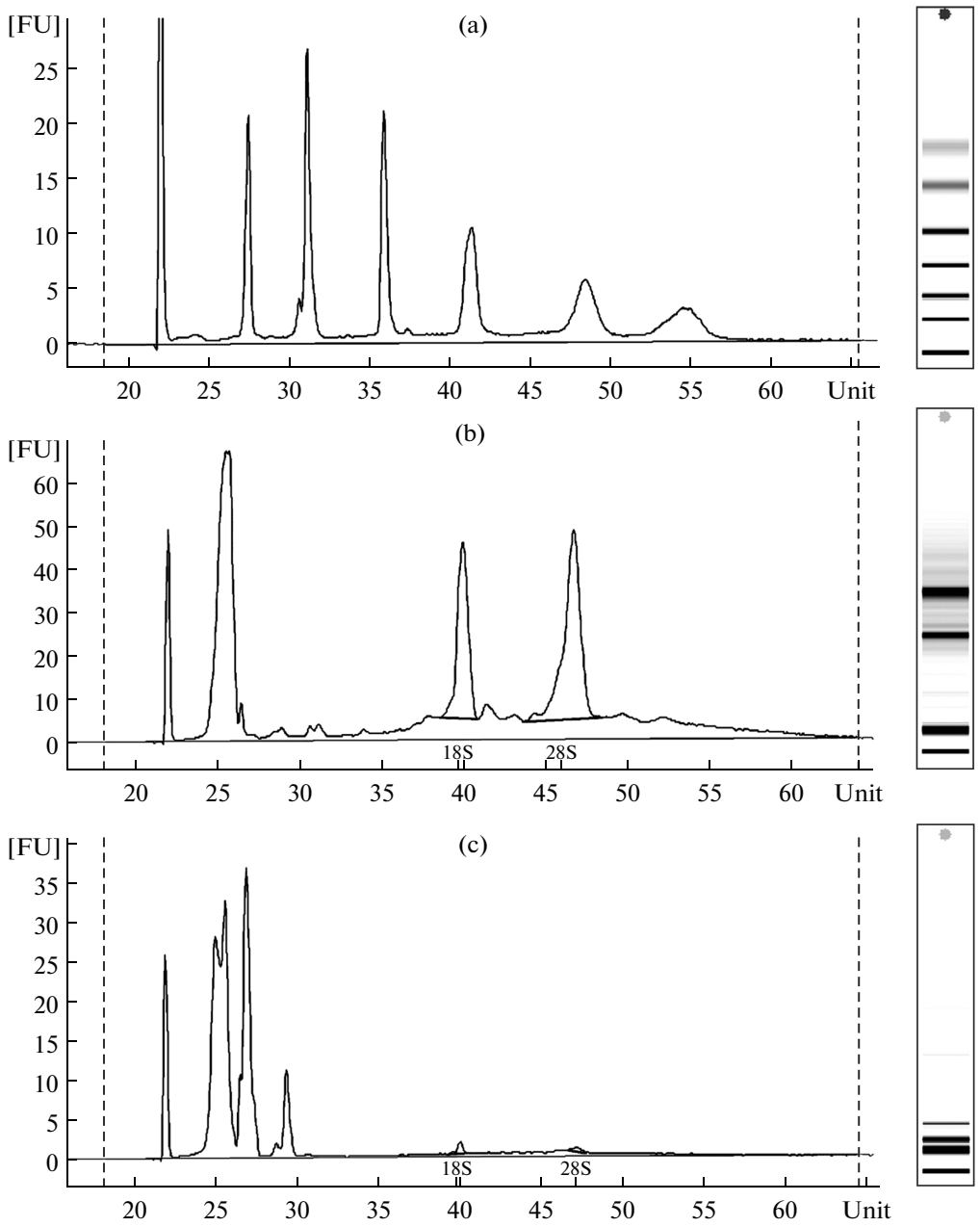

Fig. 1. Comparison of total RNA profiles from the cytoplasm and microsurgically isolated nuclei from growing chicken oocytes. The control DNA ladder of the size markers contained the following peaks: 25, 200, 500, 1000, 2000, 4000, and 6000 nucleotides (a); chromatogram and pseudo-electrophoreogram of 9 ng of total RNA from the cytoplasm (b) and 2.6 ng of total RNA from the nucleus (c) obtained by electrophoresis in the microfluidic chip. The y axis shows fluorescence intensity, which is proportional to the amount of the RNA molecules (FU is the relative fluorescence intensity); the length of the RNA molecules is plotted along the x axis (a unit is the relative migration time). The peak positions for 18S and 28S rRNAs are marked. The RIN value calculated for the RNA sample from the cytoplasm was equal to 8.3 units, and corresponding 28S/18S rRNA ratio was 1.4.

was not less than 5.0 units (up to 8.3 units) and the ratio of 28S : 18S rRNA concentrations was not less than 1.0 (Fig. 1b).

Detailed analysis of the RNA fractions from growing chicken oocytes of different sizes revealed that oocyte growth is accompanied by the accumulation of rRNAs in the cytoplasm. It is known that the expression levels of 18S and 28S rRNAs in the oocyte decrease significantly after ovulation (Elis et al., 2008). Heterogeneous RNAs were observed across the whole spectrum of the analyzed range of the RNA size (Fig. 1b).

The profile of the total RNA from the microsurgically isolated nuclei from growing oocytes of domestic

chickens was significantly different. The major peaks corresponded to the low molecular weight RNAs, including transfer RNAs and small nuclear RNAs (Fig. 1c). We did not detect significant amounts of heterogeneous-sized RNAs that could correspond to different types of transcripts synthesized at the lampbrush chromosome stage.

It is important to note that the samples of the total RNA from the oocyte nuclei were almost completely devoid of 28S and 18S ribosomal RNAs. For this reason it was not possible to assess the integrity of the RNA samples by calculating the RIN or ratios of the amounts of the 28S : 18S rRNAs (Fig. 1c). These results confirm on a biochemical level the previously established inactivation of the single nucleolar organizer on chromosome GGA16 during oogenesis in sexually mature hens (Greenfield, 1966; Gaginskaia and Chin, 1980; Hutchison, 1987). The observed trace amounts of 28S and 18S RNAs in these preparations are most likely an admixture of the RNAfrom the cytoplasm rather than the result of nuclear genome transcription. Since RNA isolation for the oocyte nuclei and cytoplasm was carried out using the same reagents and protocol, and the cytoplasmic RNA was well preserved, we can assume that the RNA from the oocyte nuclei also did not degrade.

The profiles of the sizes of the RNAs from the oocyte nuclei in domestic chicken differed from those in Xenopus. Indeed, the nuclei of frog oocytes have been shown to contain newly synthesized 18S and 28S rRNAs, which is associated with the activity of the amplified nucleoli in the oocyte nucleus (Anderson and Smith, 1978). Note, that similar differences in the profile of high molecular weight RNAs were observed in the mouse testes and sperm (Kawano et al., 2012). The profile of the total RNA in the testes contained expressed peaks for 28S and 18S rRNAs. In contrast, the profile of the total RNA from the sperm was almost devoid of long RNAs, including ribosomal RNAs.

Thus, the presence of rRNAs in the cytoplasm, but not in the nuclei of the growing oocytes, strongly indicates that during oogenesis in chicken, and probably in other avian species, rRNAs are transferred from the follicular epithelium surrounding the oocyte. This result is consistent with a previously stated hypothesis on the role of the follicular epithelium in supplying the chicken oocyte with different types of RNAs (Gaginskaia and Chin, 1980), and data on the presence of one or more nucleoli in the follicular cells of bird ovaries (Gilbert et al. 1983; Krasikova et al., 2012).

Profile of Low Molecular Weight RNAs in Chicken Oocytes

The expression profile of short RNAs during oocyte growth in *G. g. domesticus* was compared to the expression profile of short RNAs in the cells of somatic tissues. Figure 2 represents a typical expression profile pattern for small (larger than 60 bp) and short (20 to 60 bp) RNAs in different chicken tissues (liver and ovary), and in oocytes at different stages of maturation.

According to the obtained results, short RNAs in the cytoplasm of growing chicken oocytes are presented by three clearly distinguished electrophoretic bands (Fig. 2). Quantitative analysis showed that in liver cells the total amount of short RNAs was 3.8-fold lower than in the ovaries, and 6.2-fold lower than in the cytoplasm of mature oocytes (Fig. 3a). The previous analysis of the total RNA from the chicken liver, lung, testis, and ovary via electrophoretic separation in a 15% PAA-gel revealed the presence of short RNAs sized from 20 to 30 bp only in the testis (Yang et al., 2012).

It is important to note, that the cytoplasm of small yellow follicles was enriched for short RNAs compared to the cytoplasm of nonhierarchical follicles. The amounts of short RNAs in the oocyte cytoplasm increased sharply (almost 15-fold) during oocyte maturation (from 1 to 4 mm in diameter) (Fig. 3b), which may indicate the specific process of accumulation and storage of short regulatory RNA species.

The fastest migrating (bottom) electrophoretic band, which visualized after low molecular weight RNA separation, may correspond to miRNAs. This assumption is supported by the analysis of mature miRNAs in domestic chicken deposited in the short RNAs database (miRBase, http://www.mirbase.org/cgi-bin/). The in-silico analysis of 499 chicken miRNAs present in the database at the time of this study revealed that mature miRNA sequences displayed a size of 18–25 b, and the most abundant chicken miRNAs are sized 20–21 b. It is interesting to note that most miRNA genes (312 genes, 63%) in the chicken genome are localized on ten largest chromosomes (macrochromosomes). However, these chromosomes were considered to be gene-depleted during the analysis of the distribution of protein-coding sequences (Rodionov, 1996).

The size of short RNAs present in the less rapidly migrating (upper) electrophoretic band of the RNA fraction isolated from chicken oocytes, correspond in size to the PIWI-interacting RNAs (piRNAs) and are enriched in the maturing oocytes of 2–4 mm in diameter. This kind of short noncoding RNAs are the potential candidates for the piRNAs in the chicken oocyte transcriptome, and have not been characterized previously. piRNAs are expressed in germ cells, and participate in the inactivation of transposable genome elements, and the regulation of the expression of specific genes in the ovary or the testis (Aravin and Hannon, 2008; Lau et al., 2009; Malone et al., 2009). Chicken piRNAs, and piRNA-like RNAs, have been recently characterized using the next generation sequencing technology (NGS) in the testes (Yang et al., 2012; Zhang et al., 2013), and in early embryos (Shao et al., 2012), including embryonic germ cells (Rengaraj et al., 2014), but not in growing oocytes.

Intermediate-sized short RNAs (middle electrophoretic band among short RNAs), that we detected

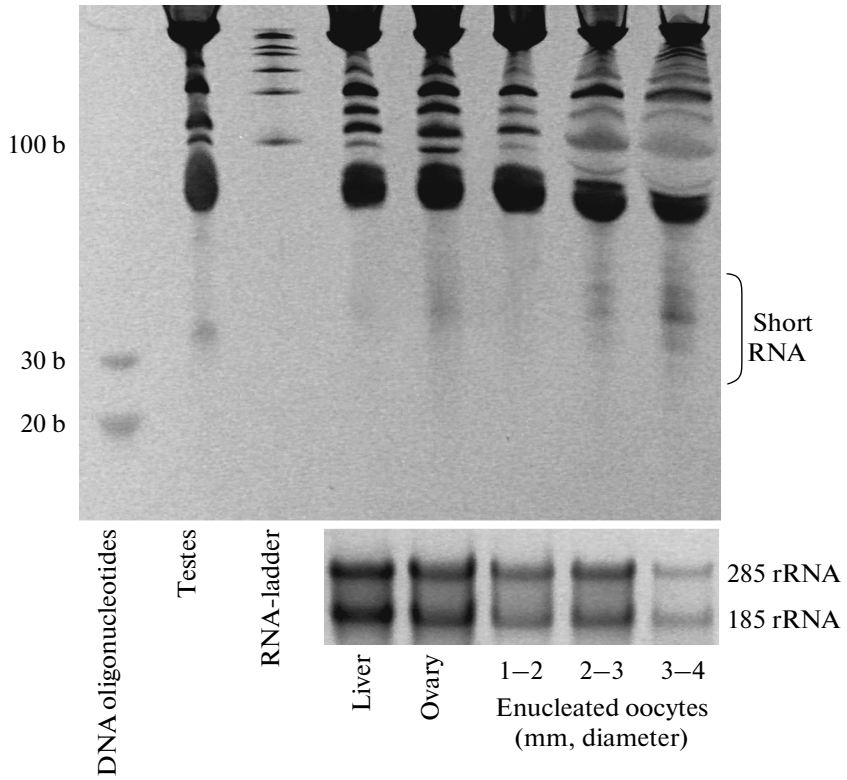

Fig. 2. The cytoplasm of maturing chicken oocytes is enriched in short RNAs. The results of the separation of short RNAs from the liver, ovary, and enucleated oocytes of domestic chicken in a 10% PAP-gel (ethidium bromide staining).

Top panel: total RNAs from the testes of Wistar rats (weighing 300 g) enriched for short piRNAs were applied as a control (lane 2). DNA oligonucleotides (lane 1) and RNA-ladder (lane 3) were used as markers of molecular weight.It is known that in 15% PAA-gel, DNA oligonucleotides migrate 10% faster than RNA oligonucleotides of the same length (Yang et al., 2012). Bottom panel: the results of separation of ribosomal RNAs from the same samples in agarose gel.

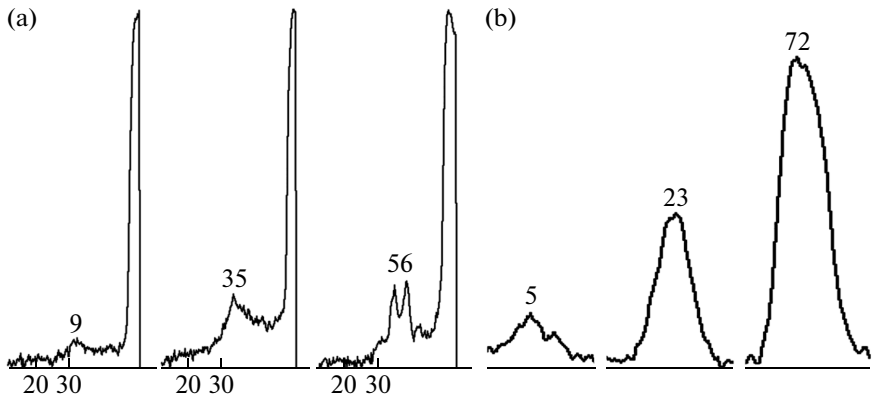

Fig. 3. Densitometry results for electrophoreograms of RNA preparations from various chicken tissues and oocyte after their separation in a PAA-gel.

(a) The expression profiles of short RNA in different chicken tissues and oocyte, left to right: liver, ovary, enucleated oocytes. The *y* axis shows the signal intensity in relative units; the *x* axis presents the length of the RNA molecules relative to the DNA oligonucleotide marker (20 and 30 b);

(b) Total amount of short RNAs at different stages of chicken oocyte maturation, from left to right: enucleated oocytes of 1–2, 2–3, and 3–4 mm in diameter. The y axis shows the signal intensity in relative units.

in the cytoplasm of enucleated chicken oocytes, are candidates for endogenous small interference RNAs (endo-siRNAs). These short RNAs are unlikely to represent molecules involved in the inactivation of retrotransposons of the LINE family (CR1 in the chicken genome), because most of the repeats of this family are

presented in the chicken genome by inactivated copies with truncated or mutated 5'UTR promoters, and their expression does not depend on the RNA interference pathway initiated by the RNase III Dicer (Lee et al., 2009).

We hypothesize that the generation of endogenous siRNAs in the cells of *G. g. domesticus* may be achieved by the processing of long double-stranded RNAs, or RNA structures containing hairpins, which can be produced by the transcription of LTR-retrotransposons and satellite DNA repeats. Such short siRNAs have been described in mouse oocytes (Watanabe et al., 2006). Earlier, we reported that tandem repeat sequences are actively transcribed from lampbrush chromosomes in chicken oocytes, and the transcription of certain DNA repeats (CNM, PO41) is carried out from both strands, which can lead to the generation of complementary double-stranded RNAs (Krasikova et al., 2006; Deryusheva et al., 2007). We proposed that transcripts of tandem and dispersed DNA repeats synthesized in avian oocytes at the lampbrush chromosome stage are processed to form short regulatory RNAs (probably, siRNAs and piRNAs). The resulting short regulatory RNAs accumulate in the oocyte cytoplasm and can play a role during the early stages of embryogenesis.

The main function of these regulatory RNAs of maternal origin is the regulation of the embryonic genome based on the mechanism of cotranscriptional silencing. In this process, the short regulatory RNAs of maternal origin can participate in the formation of heterochromatin in the absence of their own regulatory transcripts synthesized in the embryo. Indeed, short regulatory RNAs have been detected at the earliest stages of embryogenesis in domestic chickens (Shao et al., 2012). Thus, the short regulatory RNAs transferred with the cytoplasm in the avian oocyte may serve as a kind of epigenetic determinants for the transfer of epigenetic information in a number of generations.

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CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest.

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