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REGULAR ARTICLE

On some structural and evolutionary aspects of rDNA amplification in oogenesis of *Trachemys scripta* turtles

Asya Davidian[1] · Elena Koshel[2] · Alexander Dyomin[1,3] · Svetlana Galkina[1] · Alsu Saifitdinova[4] · Elena Gaginskaya[1]

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Abstract

The features of rDNA amplification have been studied in oocytes of the red-eared slider *Trachemys scripta* using a number of specific histochemical and cytomolecular methods. A single nucleolus in early diplotene oocytes is associated with the nucleolus organizer region (NOR). With oocyte growth, the number of nucleoli increases dramatically and reaches hundreds by the lampbrush chromosome stage (pre-vitellogenesis). RNA-polymerase I, fibrillarin, and PCNA immunodetection in the amplified nucleoli and FISH of the 5'ETS probe to the oocyte nuclear content suggest pre-rRNA and rDNA synthesis in the nucleoli at all stages studied. This implies a continuous reproduction of the nucleoli during oocyte development from early diplotene up to vitellogenesis. The data obtained offer a different way for rDNA amplification and formation of extrachromosomal nucleoli in turtle oocytes compared with the amplified nucleoli formation in amphibian and fish oocytes. In the Sauropsida clade of Archelosauria, which includes turtles, crocodiles, and birds, rDNA function is known to be suppressed in avian oogenesis during the lampbrush stage (Gaginskaya et al. in Cytogenet Genome Res 124:251–267, 2009).

Keywords Reptilia · Red-eared slider · Oocytes · Extrachromosomal nucleoli · rDNA amplification

Introduction

Oogenesis is a particular type of cell differentiation responsible for the formation of a mature egg containing a maternal stock of macromolecules to ensure initial embryo development. Among others, a lot of ribosomal RNA (rRNA) are required to create an apparatus for massive protein synthesis in the embryo cells, which rapidly increase in number during cleavage and blastula formation, as was studied in detail in amphibian embryogenesis (in particular Davidson 1986). During the early development of *Xenopus laevis*, the nucleolus organization is not associated with the transcription process per se but rather with the presence of maternal unprocessed rRNAs (Verheggen et al. 1998, 2000). The source of the maternal rRNA can be endogenous (a function of the oocyte inherent genome) or exogenous (a function of the genomes inherent to associated cells). Respectively, the peculiarity of a nucleolus organizer (NOR) functionality in the oocyte genome and the origin of the maternal rRNA stockpile in the oocyte define some important features related to the oogenesis type. Hypertranscriptional oogenesis (Dondua 2018) is often accompanied by the amplification of NOR ribosomal DNA (rDNA) resulting in the formation of multiple extrachromosomal nucleoli in the oocyte nucleus (germinal vesicle, GV). This is typical of oogenesis in many animals, both invertebrates and vertebrates. Such animals do not necessarily belong to close taxa. For example, among fish, NOR rDNA amplification is typical of Acipenseridae (Raikova 1976) and teleost fish (Vincent et al. 1969; Thiry and Poncin 2005), yet apparently not of Elasmobranchii like sharks and skates (Rückert 1892; Diaz-Andrade et al. 2011).

In vertebrates, apart from certain fish, rDNA amplification has been described in oocytes of all amphibians (Macgregor 1972, 1982; Callan 1986; Davidson 1986) and some reptilians (Macgregor and Klosterman 1979; Macgregor 1982), while avian oocytes with well-developed lampbrush chromosomes have been found to lack amplified nucleoli (Gaginskaya and Gruzova 1969; Gaginskaya 1972; Gaginskaya et al. 2009;

✉ Elena Gaginskaya
elena.gaginskaya@spbu.ru

[1] St Petersburg University, St Petersburg 199034, Russia

[2] ITMO University, St Petersburg 197101, Russia

[3] Saratov State Medical University, Saratov 410000, Russia

[4] Herzen State Pedagogical University of Russia, St Petersburg 191186, Russia

Koshel et al. 2016). In birds, the huge stockpile of rRNA in the oocyte is of exogenous origin, being supplied by follicular cells within specific organelles called "transosome" (Press 1964). The latter constitute a kind of ribosome-filled vesicles separating from the processes of follicular cells into the oocyte (Bellairs 1965; Press 1964; Schjeide et al. 1970, 1975). In mammalian oocytes, rDNA does not appear to be amplified either (Tian et al. 2001).

The phenomenon of rDNA amplification has been studied most thoroughly in amphibian oocytes, where the meiotic extra synthesis of rDNA results in the formation of the so-called nuclear DNA cap at the pachytene stage of the meiotic prophase (Brown and Dawid 1968; Gall 1968; Macgregor 1968, 1972; Perkowska et al. 1968; Gall and Pardue 1969; Ficq and Brachet 1971). At the early diplotene stage, rDNA copies dissociate from the cap to form thousands of extrachromosomal nucleoli predominantly active in the period of early vitellogenesis (Macgregor 1972; Spring et al. 1996). These multiple extrachromosomal nucleoli are located on the periphery of the GV. Their structure, molecular composition, and activities have been explored comprehensively (Miller and Beatty 1969; Macgregor 1972; Bakken 1975; Spring et al. 1996; Mais and Scheer 2001; Mais et al. 2002; Brangwynne et al. 2011).

Few thorough data on rDNA functioning in reptilian oogenesis are available, being mainly obtained using histological and cytogenetic approaches (Arronet 1973; Macgregor and Klosterman 1979; Guraya 1989; Callebaut et al. 1997; Uribe and Guillette 2000; Pérez-Bermúdez et al. 2012). Nevertheless, these data indicate a variety of rDNA functional activity during oogenesis in different representatives of this polyphyletic group of reptilian sauropsids (Macgregor 1982). The information appears to be contradictory to some extent. rDNA amplification has been observed in primitive reptiles of *Bipes* genus (Macgregor and Klosterman 1979) and turtles (Macgregor 1982; Callebaut et al. 1997). However, according to some authors (Hubert and Andrivon 1971; Arronet 1973; Macgregor 1982; Klosterman 1983; Guraya 1989; Vieira et al. 2010), rDNA does not amplify in lizard oocytes: a single nucleolus breaks down in the previtellogenic oocytes (Ricchiari et al. 2003) similarly to chicken oogenesis (Koshel et al. 2016; Davidian et al. 2017). Ribosomes, in enormous amounts, reach the oocyte from follicular pyriform cells via intercellular bridges and stock up within special cytoplasmic ribosomal bodies (Taddei 1972). However, Motta et al. (1991) have demonstrated DNA synthesis at the zygotene–mid-pachytene stages in the lizard *Podarcis sicula* oocytes that eventually resulted in a 5-fold increase of rRNA genes. The authors have estimated this phenomenon as a low level of rRNA gene amplification representing a small source of rRNA stockpiled in Lacertidae oocytes in addition to follicular cells. An uncertain reference to numerous nucleoli found in lizard (*Sceloporus grammicus*) GVs is also available, but these observations were made on paraffin sections stained with hematoxylin-eosin (Lozano et al. 2014). Cytological changes in the nucleolar apparatus related to the oogenesis in lizards, agamas, and some other representatives of Squamata seem to be very similar to those related to the oogenesis in chicken and, apparently, other birds (Koshel et al. 2016; Davidian et al. 2017). Essentially, lizards and birds represent phylogenetically the most distant taxa within the Sauropsida group (Crawford et al. 2012, 2015). The closest reptiles to birds, namely crocodiles and turtles, seem to have the program of NOR functionality in oogenesis completely different from both lizards and birds. Multiple peripheral bodies inside oocyte nuclei observed on ovary histological sections stained with hematoxylin-eosin are considered to be extrachromosomal nucleoli (Macgregor 1982; Guraya 1989; Moore et al. 2008; Nainan et al. 2010; Pérez-Bermúdez et al. 2012). A more detailed study of the oocyte nuclear structures in the red-eared slider *Trachemys scripta* belongs to M. Callebaut, who described numerous peripheral pyroninophylic nucleoli in the lampbrush stage oocytes and their accumulation in the center of the GV to form a karyosphere in pre-mature oocytes (Callebaut et al. 1997). However, the pattern of nucleoli multiplication in reptilian sauropsid oocytes remains somewhat unsettled.

In this paper, we have used a series of specific histochemical and cytomolecular approaches to investigate the nature, composition, and dynamics of extrachromosomal nucleoli in the oocytes of the red-eared slider *T. scripta*. Oocytes from the early diplotene up to lampbrush stage have been analyzed. We have distinguished extrachromosomal nucleoli from numerous coilin-containing nuclear bodies and demonstrated rDNA replication to take place inside the extrachromosomal nucleoli. Specific features of rDNA amplification strategy in oocytes of the red-eared slider have been described and compared with the same strategy in amphibian and fish oocytes.

Materials and methods

Biological materials and ethical approval

Nucleoli from oocytes of the red-eared slider *T. scripta* were explored. The ovaries were obtained from six 7-year-old mature females and two 1-year-old immature females. The procedures related to manipulation of animals were approved by the Ethical Committee of St. Petersburg State University (Statement #131-03-3 issued 01.06.2017) in accordance with the NIH guidelines set forth in Guide for the Care and Use of Laboratory Animals (2011).

Ovary cryosections

The pieces of *T. scripta* ovaries were fixed at room temperature in 4% paraformaldehyde in PBS for 2 h, washed several times in PBS, cryoprotected with 30% sucrose in PBS overnight at + 4 °C, frozen in Surgipath® FSC 22® Frozen Section Embedding Medium (Leica Biosystems, USA) in liquid nitrogen, and stored at − 80 °C. Cryosections of 10 or 20 μm made using Leica CM1850UV cryotome (Leica Biosystems, USA) were mounted on Superfrost-plus slides (Thermo Scientific, Germany). Before processing, the cryosections were dried for 2 h at room temperature.

Manual dissection of GV and GV content from oocyte

Oocyte nuclei (GVs) and their inner contents were manually isolated from the lampbrush oocytes of 0.5–1.5 mm diameter using Leica MZ12 stereomicroscope and tungsten preparative needles. The procedures were carried out in "5:1 + phosphates" medium according to the previously described protocol (Saifitdinova et al. 2017). GVs were stained with SYTOX Green (Molecular Probes, USA), nucleic acid specific fluorochrome, or with SYBR Green (Molecular Probes, USA), double-stranded DNA (dsDNA) specific fluorochrome, diluted to 1:5000 and 1:1000 in "5:1 + phosphates" medium, respectively.

Indirect immunofluorescent staining

Immunofluorescent staining was applied to ovary cryosections, whole isolated GVs, and GV content spreads from lampbrush stage oocytes. Indirect immunostaining procedure was carried out as described previously (Davidian et al. 2017). To minimize the nonspecific antibody binding, the slides were incubated in 5% Gibco horse serum (ThermoFisher Scientific, USA) in PBS for 1 h at + 37 °C.

The primary anti-fibrillarin antibodies (ab4566, Abcam, United Kingdom), RNA-polymerase I (Ochs et al. 1994), dsDNA (MAB030, Chemicon International, USA), PCNA (ab29[pc10], Abcam, United Kingdom), FLASH (Yang et al. 2009), and anti-p80 coilin polyclonal serum R288 (Andrade et al. 1991) were used at a dilution of 1:500, 1:100, 1:300, 1:1000, 1:100, and 1:2000, respectively. The ovary cryosections, intact GVs, and GV spreads were incubated with primary antibodies overnight at + 4 °C, and with the corresponding secondary antibodies for 1 h at + 37 °C. All antibodies were diluted in PBS with 5% Gibco horse serum (ThermoFisher Scientific, USA). The preparations were counterstained with 1 μg/mL DAPI (4′, 6-diamidino2-phenilindole-dihydrochloride) in DABCO (1,4-diazabicyclo[2.2.2]octane) antifade solution in PBS with glycerol.

FISH probe preparation

To prepare FISH probe of 5′external transcribed spacer (5′ ETS), the PCR primers were designed from the de novo assembled rRNA gene cluster of *T. scripta*, using the Unipro UGENE 1.16.1 software package, as follows: F 5′-GGTC GCTGACTTCTTCTCTA and R 5′-AAGAAGGATGTCGG GAGTC (Beagle Ltd., Russia). The probe was amplified and labeled with digoxigenin by PCR using these primers. The reaction mixture contained 1×*Taq* buffer with 2.5 mM $MgCl_2$ (Sileks, Russia), 0.07 mM digoxigenin-11-dUTP (Jena Bioscience, Germany), 0.4 mM dATP, dCTP, and dGTP, 0.13 mM dTTP (Sileks, Russia), 0.4 μM F and R-primers, 2.5 U *Taq* polymerase (Sileks, Russia), and 10 ng *T. scripta* genomic DNA per 20 μL. The PCR was performed in a MJ Mini (BioRad, USA) amplifier. PCR protocol: 5 min at 94 °C; 35 cycles of 20 s at 94 °C, 15 s at 57 °C, and 20 s at 72 °C; 5 min at 72 °C; and hold at + 4 °C.

The 5′ETS sequence is spliced from the pre-rRNA molecule when it maturates to 18S, 5.8S, and 28S rRNA. 5′ETS RNA does not incorporate into ribosomes, being present only in the nucleolus. This makes it a reliable marker of pre-rRNA transcripts when used in accordance with the RNA FISH protocol.

Oligonucleotide probe, 5′-CGCGUUCUCUCCCUCUCA CUCCCCAA-Cy3, specific to U3 snoRNA, was also used as an RNA probe for RNA FISH. This probe was kindly provided by I. Aparin (Shemyakin-Ovchinnikov Institute of bioorganic chemistry, Russia).

Fluorescent in situ hybridization

FISH was applied to ovary cryosections and GV content spreads. The cryosections were pre-treated similarly to fluorescence immunostaining, while the GV content spreads were not pre-treated. FISH was performed as described earlier in Davidian et al. (2017). For DNA in situ hybridization, the preps were denatured and pre-treated with RiboShredder™ RNase Blend (Epicentre Biotechnologies, USA) at a dilution of 1:10 for 1 h at + 37 °C. For RNA FISH, the preps were neither denatured nor pre-treated with RNase. To reduce nonspecific antibody binding, the preps were incubated in 5% Gibco horse serum (ThermoFisher Scientific, USA) solution in 4×SSC with 0.1% Tween-20 for 1 h at + 37 °C. To detect digoxigenin-labeled probe, primary and secondary antibodies conjugated with cyanine Cy3 (Jackson ImmunoResearch, USA) were used at a dilution of 1:400 for 1 h at + 37 °C with subsequent washing in 4×SSC with 0.1% Tween-20, and 2×SSC. The slides were counterstained with DAPI in DABCO antifade solution with glycerol and 2×SSC.

Microscopy

The slides were examined using DMRXA and DM4000B (Leica Microsystems, Germany) epifluorescence microscopes and Leica TCS SP5 (Leica Microsystems, Germany) confocal laser scanning microscope with the related software. The finished image was produced using the maximum intensity projection function. The lasers used for analysis were 488 nm and 543 nm. Confocal microscope images were processed, deconvoluted, and analyzed with SVI Huygens software. To quantify the nucleoli number inside the GV, Fiji software using the "3D objects counter" function was applied to the manually isolated GVs immunostained with anti-fibrillarin antibodies. In total, eight GVs from the lampbrush oocytes were analyzed.

Results

According to morphological data (Callebaut et al. 1997), the ovaries of 1-year immature turtle *T. scripta* contain oocytes from the leptotene stage to the pre-lampbrush diplotene meiotic stage. The lampbrush and post-lampbrush oocytes are present in the ovaries of older immature and adult females (Guraya 1989; Callebaut et al. 1997). In this work, we investigated the contents of the nucleus in oocytes from the ovaries of both immature and adult females non-stimulated to oocyte maturation. In our study of the features of amplified nucleoli origination and development in *T. scripta* oocytes, we focused on three successive stages of oocyte growth, namely, earlier diplotene oocytes located in germinal beds and not surrounded by follicular cells, pre-lampbrush diplotene oocytes, which are already surrounded by follicular epithelium, and the larger oocytes with well-developed lampbrush chromosomes in the GV. Nuclear structures were explored using ovary cryosections, both intact GV and GV content manually dissected from the oocyte. For nucleoli detection at different oocyte stages, both in situ hybridization of the 5'ETS probe and immunofluorescent detection of antibodies specific to fibrillarin were used. Notably, GV and GV content manual dissection is possible from the lampbrush oocytes only.

Nucleoli and coilin-containing bodies can be detected at all analyzed stages of *T. scripta* oocyte growth

In GVs manually dissected from oocytes of 0.5–1.5 mm diameter, multiple round nuclear bodies were observed after staining with SYTOX Green (Fig. 1a). On a maximum intensity confocal projection, lampbrush chromosomes are distinguishable, and extrachromosomal nuclear bodies of different sizes are brightly fluorescent. The largest bodies can be vacuolized. It is commonly known that amphibian GVs contain a good deal of amplified extrachromosomal nucleoli along with various coilin containing bodies, such as Cajal Bodies (CB), Histon Locus Bodies (HLB), pearls (Nizami et al. 2010; Nizami and Gall 2012). To distinguish nucleoli from extrachromosomal coilin-containing bodies, we applied simultaneous immunostaining with fibrillarin and p80 coilin antibodies. In lampbrush oocytes, numerous extrachromosomal bodies were found to be true nucleoli, while the rest represented coilin-containing entities (Fig. 1b–b"). This confirmed the previous hypotheses of rDNA amplification in turtle oogenesis (Guraya 1989; Callebaut et al. 1997).

In lampbrush oocytes, coilin-containing bodies had different configuration and size: some of them had a round shape (Fig. 2a), while others resembled rings varying in size (Fig. 2b). Noteworthy is that only one type prevails in each lampbrush oocyte. In some GVs, we observed irregular ring-like coilin-containing bodies of a diameter exceeding 10 μm. The coilin-containing bodies of all types were not found to interact with anti-FLASH antibodies after indirect immunostaining (not shown). Most likely, they are CBs and/or some unidentified bodies rather than HLB. In the coilin-containing bodies, we never detected fibrillarin, while the nucleoli never revealed coilin. The nucleoli are clearly identifiable when immunostained with anti-fibrillarin antibodies (Fig. 2a) or hybridyzed with U3 snoRNA probe (Fig. 2b). The U3 snoRNA was shown to participate in the earliest cleavage event of pre-rRNA processing and remain bound to the processed rRNA product (Kass et al. 1990; Correll et al. 2019).

Early diplotene oocytes situated within the ovary germinal beds comprise 1 or 2, sometimes 3, nucleoli (Fig. 3a), and a single coilin-containing body (not shown). As oocytes grow, the number of nucleoli and coilin-containing bodies increases. The GVs in pre-lampbrush oocytes may contain a dozen or more nucleoli (Fig. 3b). In GVs with completely developed lampbrush chromosomes, the number of nucleoli and coilin-containing bodies increases, the nucleoli number reaches 300–400. On the frozen sections of the lampbrush oocytes, most of the nucleoli are located on the periphery of the nucleus directly under the nuclear envelope (Fig. 3c).

The sizes of the nucleoli in the lampbrush oocyte GVs vary significantly. The nucleoli may vary within 1–40 μm range in the same GV. Figure 4 represents a typical sample of the *T. scripta* oocyte nucleoli organization and diversity as it is visible on the GV content preps stained with anti-fibrillarin antibodies and counterstained with DAPI. The significant part of the nucleolar volume is a zone of fibrillarin location, which suggested the fibrillar component area within the bipartite nucleolus, a distinctive, presumably granular component (GC) area around it (Thiry and Lafontaine 2005; Bartholomé et al. 2019). It is noteworthy that some small fibrillarin inclusions have been found within the suggested GC area that may be very small nucleoli. At this level, we cannot say whether the smaller nucleoli merge with each other or split off the larger ones. Being non-membrane intranuclear organelles,

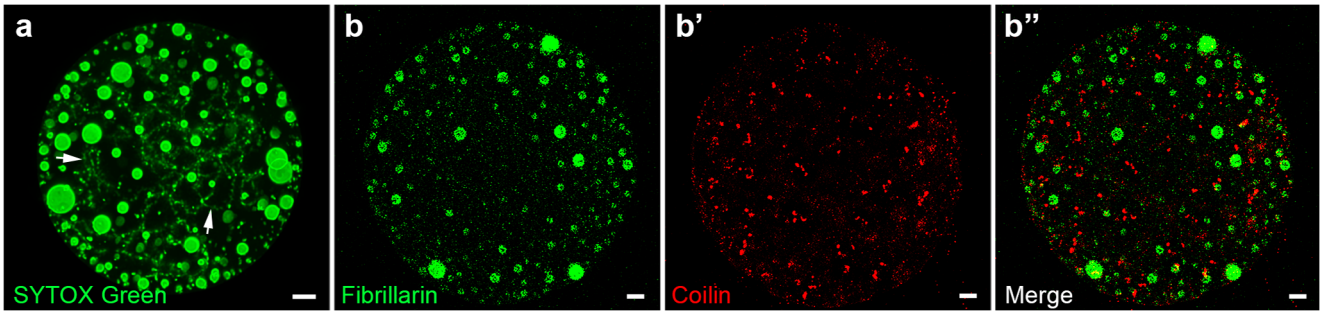

Fig. 1 Germinal vesicles isolated from *T. scripta* oocytes, lampbrush stage. **a** Lampbrush chromosomes (arrows) and undifferentiated nuclear bodies in GV, SYTOX green fluorochrome specific to DNA and RNA nucleic acids. **b–b"** Nuclear bodies discriminated using immunostaining with fibrillarin (**b**, green) and p80 coilin (**b'**, red) antibodies. **b"** Overlay. Confocal maximum intensity projections. Scale bars, 10 μm

which are phase-separated condensates, they can either merge or split (Brangwynne et al. 2011; Feric et al. 2016).

Nucleoli are active in pre-rRNA synthesis at all studied stages of *T. scripta* oocyte growth

At every studied stage of oocyte development, the nucleoli demonstrated the same pattern of nucleolar activity. This is shown by detection of the pre-rRNA nascent transcripts in nucleoli when performing RNA FISH of 5'ETS probe to the cryo-sectioned prep (Fig. 3a–a"). Figure 3 shows incompletely labeled nucleoli when RNA FISH was performed with 5'ETS probe. The fluorescence pattern was comparable to that of the fibrillarin fluorescence in Fig. 4. Although this might be in a certain inconsistency with data on the earliest separation of 5'ETS during pre-rRNA processing (Turowski and Tollervey 2015), we consider the result in accordance with the fact that in *Xenopus* oocytes maternal rRNAs are stored as unprocessed pre-rRNA (Verheggen et al. 1998, 2000). The rDNA transcription is supported by the detection of RNA-polymerase I in the nucleoli. Figure 5a–a" demonstrates the presence of RNA-polymerase I and fibrillarin in the oocyte nucleoli at early diplotene and pre-lampbrush stages. The RNA-polymerase I signal, which ought to be associated with active rDNA repeats, is scattered over the fibrillarin (suggested fibrillar component) location zone (Fig. 5a" and insertion).

The same pattern of RNA-polymerase I signal distribution was revealed at all studied stages of the *T. scripta* oocyte development. Fibrillarin and U3 snoRNAs were also found in all nucleoli, which is indicative of pre-rRNA processing and general nucleolar activity in the red-eared slider oocytes.

Amplified rDNA replicates in extrachromosomal nucleoli at all studied stages of *T. scripta* oocyte growth

In GVs manually dissected from lampbrush oocytes, the DNA-specific SYBR Green fluorochrome identified lampbrush chromosomes in the central area of the GV and manifested at least one bright DNA granule per nucleolus (Fig. 6a). DNA content in nucleoli was also detected when nucleoli isolated from GVs had been immunostained with anti-dsDNA antibody (Fig. 6b). As shown in Fig. 6b, DNA inclusions feature different patterns in different nucleoli. We show that 5'ETS probe fluorescent signal colocalizes with these DNA granules after DNA FISH (Fig. 6c–c"). It is noteworthy that larger nucleoli contain two or more DNA granules located both inside and on the surface of the nucleolus (Fig. 6b, c).

The evidence of DNA synthesis in the nucleoli was shown using indirect immunodetection of antibody against PCNA DNA replication factor at all investigated stages of oocyte growth (Fig. 7a–c). Fluorescence of the entire nucleolus is usually detected using this antibody (Fig. 7a, c) as it was described for the nucleoli of human tumors and various somatic cells (Chan et al. 1983). In any case, it appears tempting to assume that the significant increase in the number of extrachromosomal nucleoli in the GV, as the oocyte grows, is related to the constant replication of nucleolar rDNA followed by multiplication of the nucleoli. In some nucleoli, amplified rDNA copies appear to remain inside as an active rDNA, which increases the volume of the nucleolus. Nevertheless, we cannot exclude the fact the larger nucleoli may originate

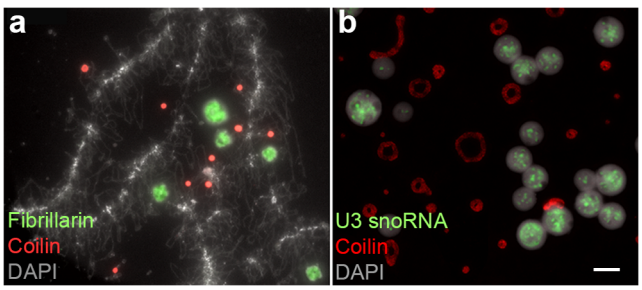

Fig. 2 Nucleoli and coilin containing bodies from a lampbrush oocyte. **a** Part of the GV content: fragments of lampbrush chromosomes (gray), extrachromosomal nucleoli (green), and round shape coilin-containing bodies (red). Double immunostaining with fibrillarin and p80 coilin antibodies. **b** Extrachromosomal nucleoli (green and gray) and irregular ring-like coilin-containing bodies (red). RNA FISH of U3 snoRNA probe to the nucleoli isolated from GV followed by immunostaining using p80 antibody. Epifluorescence microscopy. Scale bar, 10 μm

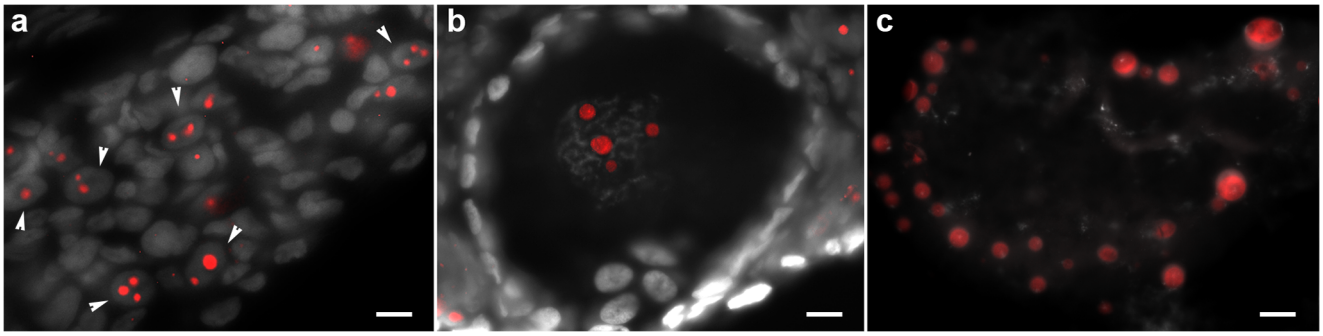

Fig. 3 Nucleoli in *T. scripta* oocytes at the successive stages of oocyte growth. **a** Early diplotene oocytes (arrowheads) in the germinal bed area of the ovary: single nucleoli (red) in the nuclei. **b** Pre-lampbrush oocyte: several nucleoli (red) in the GV. **c** Lampbrush oocyte nucleus: the nucleoli (red) on the periphery of the nucleus. RNA FISH using 5'ETS probe on cryosections, counterstaining with DAPI (gray). Epifluorescence microscopy. Scale bars, 10 μm

as the result of the fusion of smaller ones. rDNA amplification as a continuous process over an extended period of oocyte growth appears to be essential.

Discussion

The nucleolus is the most prominent non-membrane nuclear organelle of almost all eukaryotic cells, which arises in association with NOR as a result of rDNA activity during the cell cycle interphase. Being involved in many physiological and pathological processes, such as cell cycle control, DNA damage repair, cell stress and homeostasis, and cancer diseases (Grummt 2010, 2013; Ogawa and Baserga 2017; Penzo et al. 2019), the nucleolus is primarily responsible for biogenesis of the protein synthesizing apparatus (Pederson 2011; Dubois and Boisvert 2016). The compartmentalization of the processes of pre-rRNA synthesis, processing and rRNA assembling with ribosomal proteins defines the nucleolus structure. The nucleoli are known to be bipartite or tripartite (Thiry and Lafontaine 2005; Lamaye et al. 2011; Thiry et al. 2011). The latter comprise fibrillar centers (FC), each containing a single or multiple rDNA repeats, a dense fibrillar component (DFC) representing pre-rRNA processing zone, and a granular component (GC) – pre-ribosome subunits formation zone. In bipartite nucleoli, the FCs are not revealed, and active rRNA genes are dispersed within the fibrillar component (Hernandez-Verdun et al. 2010; Lamaye et al. 2011).

Our results seem to be relevant to data on bipartite nucleolus structure in turtle somatic tissues (Thiry and Lafontaine 2005; Hernandez-Verdun et al. 2010; Lamaye et al. 2011; Thiry et al. 2011; Bartholomé et al. 2019). We found the extrachromosomal nucleoli in *T. scripta* oocytes to reveal rDNA containing material scattered over the fibrillar component detected by fibrillarin staining, as well as RNA-polymerase I and U3 snoRNA unevenly distributed over the same part of the nucleolus. Pre-rRNA transcripts were revealed in the nucleoli at all studied stages of oocyte development. All the obtained data confirm functional activity of the nucleoli from early diplotene to the late lampbrush stage.

Exploration into the functional organization and dynamics of extrachromosomal structures in *T. scripta* GVs have made the concept of rDNA amplification during oogenesis in some of Sauropsida more evidence-based. Although there are no available data related to the beginning of rRNA zygotic synthesis in reptilian embryogenesis, we can assume that similarly to *Xenopus* (Davidson 1986) and chicken (Zagris et al. 1998), the embryonic genome activation in reptilians should happen somewhat about the period of middle blastula–gastrula formation. It is notable that in *Xenopus* oocytes,

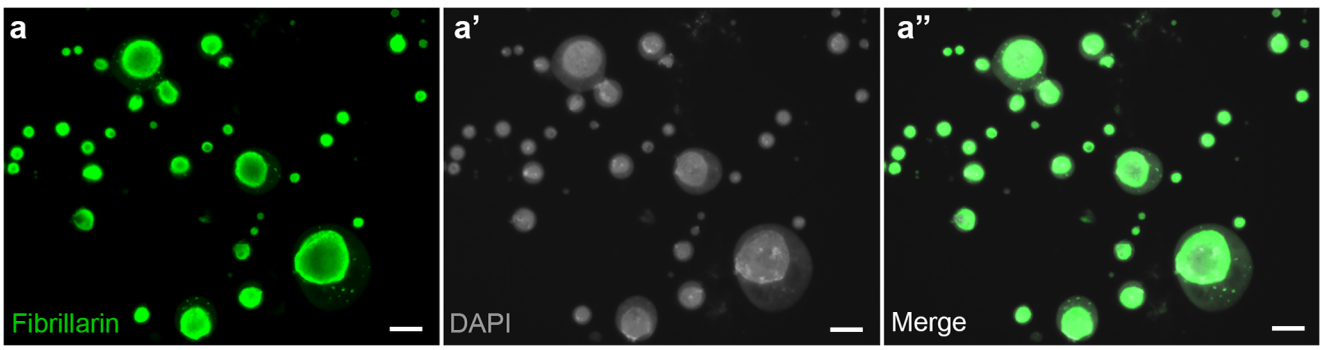

Fig. 4 Extrachromosomal nucleoli manually dissected from the *T. scripta* GV. Immunostaining with anti-fibrillarin antibodies (**a**), counterstaining with DAPI (**a'**) and merge (**a"**). Scale bars, 10 μm

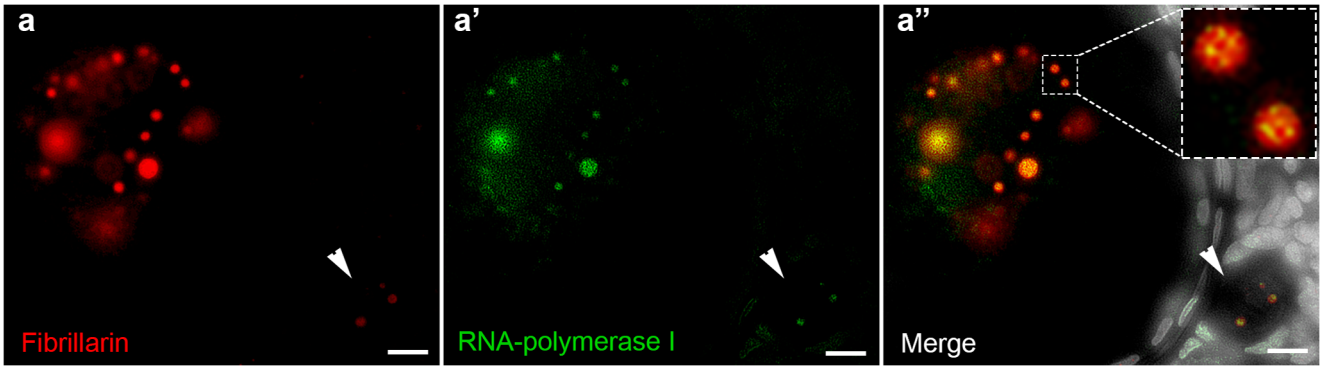

Fig. 5 RNA-polymerase I detection within nucleoli in *T. scripta* GV. **a**–**a"** Cryosection of the ovary fragment: pre-lampbrush oocyte and small early diplotene oocyte (arrowhead) in germinal bed. Double immunostaining with fibrillarin (**a**) and RNA-polymerase I (**a'**) antibodies. **a"** Overlay, DAPI channel (gray) is added. Epifluorescence microscopy. Scale bars, 10 μm

maternal rRNAs are stored as unprocessed pre-rRNA (Verheggen et al. 1998, 2000). It can be assumed that the labeling pattern of the *T. scripta* nucleolus with RNA FISH using the 5'ETS probe is due to the same oocyte feature. Anyway, NOR rDNA amplification and functioning of numerous extrachromosomal nucleoli in *T. scripta* oogenesis are definitely of a great importance for the maternal rRNA stockpiling and its endogenic source.

It seems reasonable to approach the consideration of the phenomenon of NOR ribosomal gene amplification in terms of versatility of nucleolar amplification strategies and the evolutionary aspect. In amphibian oogenesis the rDNA amplification process starts in oogonia. rDNA synthesis evolves slowly until the pachytene stage. Then, very intensive rDNA synthesis at pachytene stage leads to formation of the "nuclear cap" of DNA (Gall 1968; Macgregor 1968; Perkowska et al. 1968; Gall and Pardue 1969; Ficq and Brachet 1971; Coggins and Gall 1972). About 11 rounds of rDNA synthesis are estimated to be involved in the production of the final number of NOR copies in *X. laevis* (Perkowska et al. 1968; Coggins and Gall 1972). DNA synthesis ceases completely at the early diplotene stage and rDNA copies disperse around the nuclear envelope to form the extrachromosomal nucleoli. The same process appears to take place in fish oogenesis (Raikova 1976). In reptilians, we obviously have quite a different pattern of rRNA gene amplification. All data indicate absence of rDNA extra synthesis and "nuclear cap" formation in early meiotic oocytes. Contrarily, the smallest diplotene oocytes feature a single true nucleolus, which is undoubtedly related to a single NOR located in chromosome 14 (Cleiton and Giuliano-Caetano 2008). The number of nucleoli increases with oocyte growth and varies between 300 and 400 at the lampbrush stage. We can confidently suggest that continuous multiplication of functionally active nucleoli occurs during oocyte growth from the early diplotene stage up to vitellogenesis.

In Sauropsida, that includes birds and reptiles, NOR functionality during oogenesis can vary fundamentally at the level

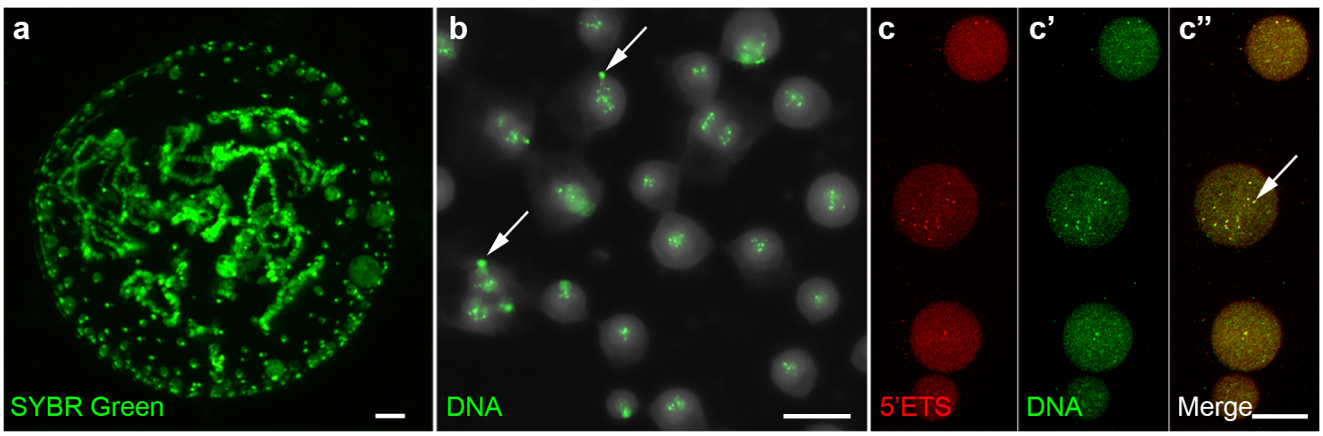

Fig. 6 rDNA detection in extrachromosomal nucleoli. **a** GV manually dissected from lampbrush stage oocyte: lampbrush bivalents and DNA granules stained with dsDNA-specific fluorochrome SYBR Green. **b** Extrachromosomal nucleoli manually dissected from the GV of lampbrush stage oocyte: DNA granules (arrows) detected using anti-dsDNA antibody, counterstaining with DAPI (gray). **c** Cryosection through the nuclear periphery of the lampbrush stage oocyte: extrachromosomal nucleoli after DNA FISH of 5'ETS probe (**c**) followed by immunostaining using anti-dsDNA antibody (**c'**), manifest a full overlay of fluorescent signals in DNA granules (arrow) (**c"**). Confocal laser scanning (**a**, **c**) and epifluorescence (**b**) microscopy. Scale bars, 10 μm

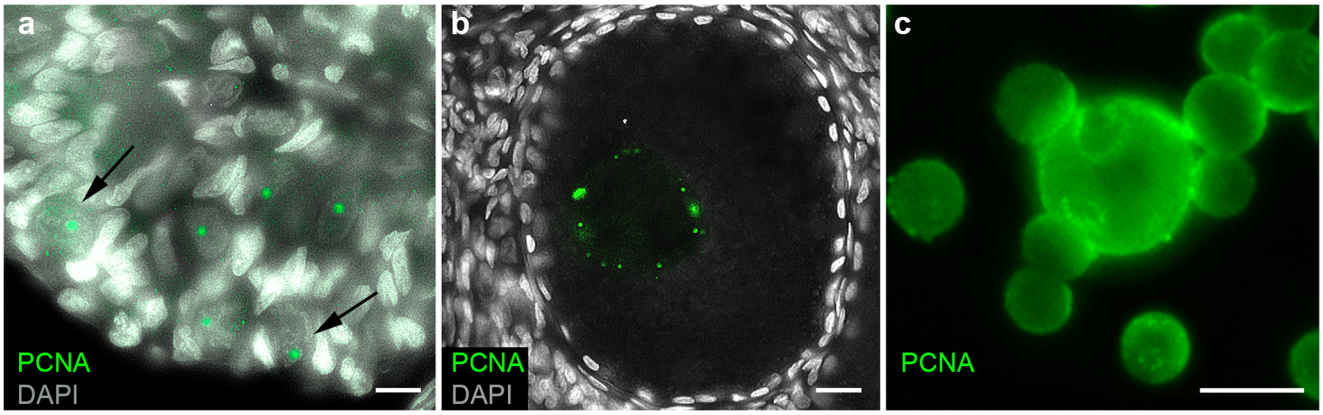

Fig. 7 DNA replication evidence in nucleoli at the successive stages of *T. scripta* oocyte growth. **a** Germinal bed zone fragment: small oocytes (arrows), presumably at the early diplotene stage. **b** Pre-lampbrush stage oocyte: extrachromosomal nucleoli of different sizes. **c** Nucleoli manually isolated from the lampbrush stage oocyte. **a**, **c** Indirect immunostaining using anti-PCNA antibody (green). **b** Overlay of PCNA (green) and DAPI (gray). Epifluorescence microscopy. Scale bar, **a** 10 μm; **b** 20 μm; **c** 10 μm

of classes, orders or even families regardless of their evolutionary proximity. Among reptilian Sauropsida, a high level of rDNA amplification could be apparently expected in all Testudines and Crocodilia (Guraya 1989; Callebaut et al. 1997; Uribe and Guillette 2000; Moore et al. 2008; Pérez-Bermúdez et al. 2012; this research). At a lower level, rDNA amplification occurs in oocytes of only some of the representatives of Squamata, such as *Bipes* genus from Bipedidae, Amphisbaenia (Macgregor and Klosterman 1979) and *Podarcis sicula* from Lacertidae, Lepidosauria (Motta et al. 1991). Absence of rDNA amplification and the peculiar pattern of nucleolar dynamics in oocytes of a vast majority of lizards and other Squamata appear to be similar to those in birds (Koshel et al. 2016; Davidian et al. 2017).

According to the recent phylogeny data (Chiari et al. 2012; Crawford et al. 2012, 2015; Liu et al. 2017), birds and crocodiles belong to a common clade Archosauria. Phylogenetically, turtles are much closer to Archosauria rather than to Squamata, and together with Archosauria are further included into a higher taxon Archelosauria. The latter evolved separately from Squamata and other reptiles (Lepidosauria). Based on the above, we can assume, actually following H. Macgregor (1982), that, since rDNA amplification occurs during oogenesis similarly in all amphibians, it is arguable that the first Sauropsida, like their ancestors, could have inherited and retained this feature (Fig. 8). Indeed, among representatives of the evolutionarily ancient branch Archelosauria (turtles and Archosauria) that formed at the beginning of the Triassic period, the phenomenon of rDNA amplification is rather common and, apparently, affects representatives of the entire orders Testudines and Crocodilia. Another point is that their rDNA amplification strategy differs from that of amphibians. The amplification phenomenon was also observed in some primitive Squamata (suborder Amphisbaenia), which form an evolutionarily distant from Archelosauria branch of Sauropsida. At the same time, among young, evolutionarily progressive taxa both in the Archelosauria branch (the entire Aves class) and the Squamata (probably the vast majority of the Serpentes and Lacertilia suborders), rDNA amplification does not occur during oogenesis. It is replaced by a more complex process of ribosome accumulation involving the biosynthetic potential of the follicular cells that source the necessary amount of rRNA to the ooplasm. Notably, there is a difference between the ways of follicular donation in birds and lizards mentioned above.

The problem deserving attention concerns the significant diversity of the rDNA amplification methods and of the ways of extrachromosomal nucleoli formation among different organisms. The nuclear cap of rDNA during the pachytene stage in amphibians and fish, and the progressive nucleoli reproduction during oocyte growth in the turtle are the brightest instances of different amplification strategies in vertebrates (Fig. 8).

The same applies to the method of delivering material from follicular cells to the ooplasm: intercellular bridges in representatives of Squamata vs the transosomes in birds and, probably, some turtles (Rahil and Narbaitz 1973). The phenomena determining the diversity of the ways of ribosomal gene amplification is a matter of fundamental importance. The question whether and how the way of nucleolar amplification may affect oocyte maturation remains open.

Concluding remarks

The experimental data obtained here confirm the amplification of ribosomal RNA genes in turtle oocytes, show the specific way of forming extrachromosomal nucleoli in their GVs, and allow us to make a series of evolutionary generalizations based on our own data and available publications. In line with Herbert Macgregor views (Macgregor 1982), we can suggest that rDNA amplification and nucleolus formation during oogenesis in reptiles is an ancient primitive mechanism of

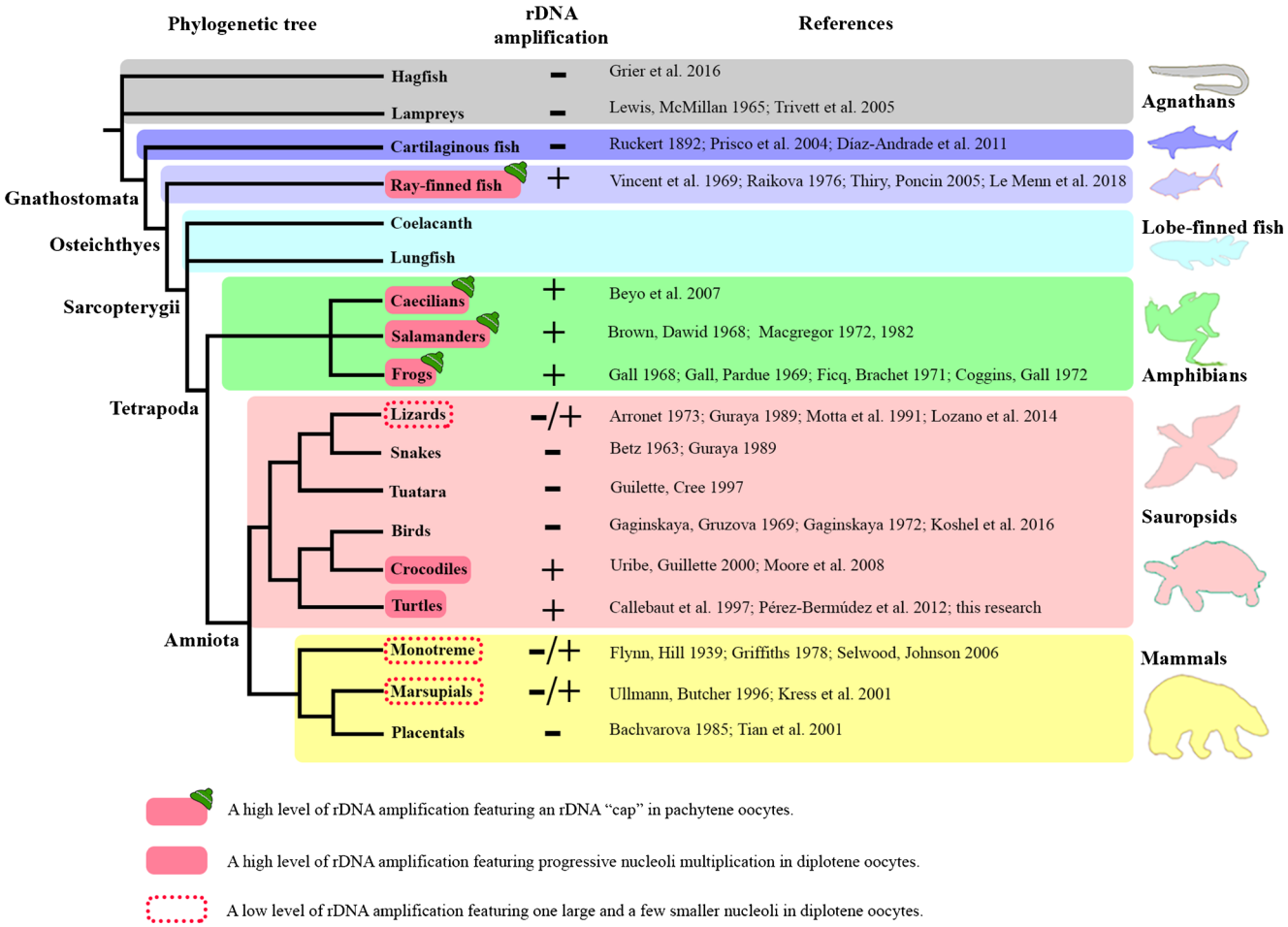

Fig. 8 rDNA amplification in oocytes among vertebrates. Nuclear rDNA cap at the pachytene stage vs continuous nucleolar reproduction in growing oocytes among some vertebrate groups. Phylogenetic tree taken from Meyer and Zardoya 2003

ribosome production increasing inherited by reptiles from amphibian ancestors. In the process of evolution, this strategy is replaced by more efficient mechanisms in various phylogenetic lines of reptiles based on the use of somatic cells surrounding the oocyte as donors. Remarkably, this transition occurs independently in the distant evolutionary lines of Squamata and Archosauria and is associated with the development of large, evolutionarily plastic, and widespread taxa (such as Aves and Lacertilia). Integration of the existing and new data on the features of rDNA amplification in reptile oocytes demonstrates the existence of a clear macroevolutionary trend in oocyte strategies related to stockpiling of an increased rRNA reserve. The reasons for the diversity of ways to amplify ribosomal genes (the nuclear rDNA cap at the pachytene stage vs continuous nucleolar reproduction in growing oocytes) remain unclear.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed were in accordance with the ethical standards of the Ethical Committee of St. Petersburg State University (Statement #131-03-3 issued 01.06.2017) and Guide for the Care and Use of Laboratory Animals (2011).

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