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Nucleolus-like bodies of fully-grown mouse oocytes contain key nucleolar proteins but are impoverished for rRNA

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ABSTRACT

It is well known that fully-grown mammalian oocytes, rather than typical nucleoli, contain prominent but structurally homogenous bodies called "nucleolus-like bodies" (NLBs). NLBs accumulate a vast amount of material, but their biochemical composition and functions remain uncertain. To clarify the composition of the NLB material in mouse GV oocytes, we devised an assay to detect internal oocyte proteins with fluorescein-5-isothiocyanate (FITC) and applied the fluorescent RNA-binding dye acridine orange to examine whether NLBs contain RNA. Our results unequivocally show that, similarly to typical nucleoli, proteins and RNA are major constituents of transcriptionally active (or non-surrounded) NLBs as well as of transcriptionally silent (or surrounded) NLBs. We also show, by exposing fixed oocytes to a mild proteinase K treatment, that the NLB mass in oocytes of both types contains nucleolar proteins that are involved in all major steps of ribosome biogenesis, including rDNA transcription (UBF), early rRNA processing (fibrillarin), and late rRNA processing (NPM1/nucleophosmin/B23, nucleolin/C23), but none of the nuclear proteins tested, including SC35, NOBOX, topoisomerase II beta, HP1α, and H3. The ribosomal RPL26 protein was detected within the NLBs of NSN-type oocytes but is virtually absent from NLBs of SN-type oocytes. Taking into account that the major class of nucleolar RNA is ribosomal RNA (rRNA), we applied fluorescence *in situ* hybridization with oligonucleotide probes targeting 18S and 28S rRNAs. The results show that, in contrast to active nucleoli, NLBs of fully-grown oocytes are impoverished for the rRNAs, which is consistent with the absence of transcribed ribosomal genes in the NLB mass. Overall, the results of this study suggest that NLBs of fully-grown mammalian oocytes serve for storing major nucleolar proteins but not rRNA.

Introduction

Growth of mammalian oocytes is a long-term process that is accompanied by remarkable functional and structural reorganizations of the nucleolus – the major multifunctional nuclear domain that plays key roles in ribosome biogenesis (Dundr, 2012; Hernandez-Verdun et al., 2010; Shaw and Brown, 2012; Grummt, 2013). In addition to RNAs (mainly, rRNA and snoRNAs), ribosome biogenesis requires numerous protein factors to ensure rDNA transcription, rRNA processing, and the export of ribosomal particles to the cytoplasm (Cisterna and Biggiogera, 2010). A remarkable feature of the mammalian nucleolus is its functional and morphological divergence that is manifested in various types of cells including oocytes. In this way, nucleoli of growing mammalian oocytes are typical active nucleoli: they contain numerous small fibrillar centers (the harbors of inactive rDNAs and RNA polymerase I), the dense fibrillar component (the site of rRNA synthesis and processing), and a granular compartment that is comprised of maturing ribosomal particles (Fair et al., 2001). Suppression of oocyte growth in antral follicles is accompanied by downregulation of the nucleolar synthetic activity and initiates transformation of the nucleoli into unique structures that are called "nucleolus-like bodies" (NLBs), or "post-nucleoli" (Chouinard, 1971). In the fully-grown (or germinal vesicle, GV) oocytes NLBs are seen as prominent, large (up to 10 μm in diameter), spherical bodies whose mass is composed of a tightly and uniformly packed fibrous material. This mass is deprived of any

Abbreviations: NLB, nucleolus-like body; NSN, non-surrounded nucleolus; SN, surrounded nucleolus; GV, germinal vesicle; MII, metaphase II; rDNA, ribosomal DNA; rRNA, ribosomal RNA; pre-rRNA, precursor rRNA; snoRNA, small nucleolar RNA; ssRNA (ssDNA), single-stranded RNA (DNA); dsRNA (dsDNA), double-stranded RNA (DNA); AO, acridine orange; FITC, fluorescein-5-isothyocyanate; RNase A, ribonuclease A.

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morphologically defined counterparts of somatic nucleoli, so that solitary fibrillar centers, the poorly developed dense fibrillar component, and abandoned ribosomal particles are only observed at the NLB surface (Antoine et al., 1988; Biggiogera et al., 1994; Longo et al., 2003). In the vast majority of GV oocytes, only one NLB is present, but other GV oocytes contain two or three NLBs, which are similar in structure and immunochemical properties but may differ in size.

Two major types of NLBs have been described in fully-grown oocytes of all mammals studied so far: the NLBs that associate with discrete blocks of heterochromatin (so-called "non-surrounded nucleoli", NSN), and the NLBs that are surrounded by a layer of heterochromatin (so-called "surrounded nucleoli", SN) (Debey et al., 1993; Zuccotti et al., 2002; Bellone et al., 2009; Tan et al., 2009). It is accepted that only the NSN-oocytes are able to synthesize rRNA, whereas the SN-type oocytes are transcriptionally silent (Bouniol-Baly et al., 1999; Pesty et al., 2007). However, in contrast to somatic nucleoli, active ribosomal genes have been described only at the NLB surface (Bouniol-Baly et al., 1999; Pesty et al., 2007). SN-oocytes have higher meiotic and developmental competence than NSN-oocytes and correspond to a more advanced stage of oocyte development (Zuccotti et al., 2002, 2011; Inoue et al., 2008). Recent analysis of the global transcriptome profile in mouse NSN- and SN-oocytes showed that they are very similar but not identical: NSN-oocytes are enriched in methylated and acetylated peptides (Monti et al., 2013), whereas SN-oocytes are characterized by higher levels of methylation and acetylation of DNAs (Kageyama et al. 2007). However, expression of nearly 30 genes encoding ribosomal proteins is upregulated in SN-oocytes compared to NSN-oocytes (Monti et al., 2013).

NLBs are assembled in fully-grown oocytes of mammals of various species, including mouse, rat, pig, cattle, and human (Chouinard, 1971; Antoine et al., 1988; Kopecny et al., 1996; Hyttel et al., 2001; Parfenov et al., 1989), but their direct homologs have not been described in other animals. These facts point to particular importance and conservative role(s) of NLBs in mammalian oogenesis. In addition, the NLB material is also indispensable for the early steps of embryonal development. Zygotic embryos originating from enucleolated oocytes are incapable of forming nucleolar precursor bodies (NPBs), have severe defects in spatial arrangement of chromatin, contain reduced amounts of the major and minor satellite DNAs, and finally become arrested at the two-cell stage (Ogushi et al., 2008; Ogushi and Saitou, 2010; Inoue et al., 2011; Fulka and Langerova, 2014). However, the reason for the NLB requirement for oogenesis and early embryogenesis remains poorly studied.

Numerous cytochemical, autoradiographic, and immunocytochemical studies have been conducted to determine the biochemical composition of the material comprising the NLB mass. Overall, the results of these studies demonstrate that: (1) NLBs do not contain polysaccharides, lipids, or DNAs (Antoine et al., 1988; Kopecny et al., 1995, 1996). (2) NLBs probably contain certain amounts of nuclear RNAs, but the results obtained by different methods are contradictory (Antoine et al., 1989; Kopecny et al., 1996). (3) NLBs most likely contain proteins (Antoine et al., 1988). However, no nucleolar proteins have been revealed within the NLB mass under conventional conditions of immunolabeling of GV oocytes (Zatsepina et al., 2000; Fair et al., 2001; Bjerregaarde et al., 2004; Romanova et al., 2006; Maddox-Hyttel et al., 2007; Pochukalina and Parfenov, 2008; Fulka and Langerova, 2014). Recently, an antigen retrieval achieved by oocyte spread boiling in sodium citrate has shown that NLBs of mouse GV oocytes contain nucleolar proteins (Fulka and Langerova, 2014), but the question whether they are present in the NLBs of both GV-type oocytes remains open. (4) Data on immunoelectron microscopy of NLBs are rather limited or contradictory. For instance, the nuclear splicing factor SC35 has been detected in the NLB mass by some authors (Kopecny et al., 1996) but not by others (Pochukalina and Parfenov, 2008). Overall, survey of the literature data shows that the biochemical composition of NLBs in fully-grown mammalian oocytes remains largely undetermined. Lack of such data makes it difficult to establish the role(s) of NLBs in oogenesis and to explain why the NLB material is required for early development of mammalian embryos.

In this study, to elicit the composition and putative functions of mammalian NLBs we optimized conditions for staining mouse paraformaldehyde-fixed oocytes with acridine orange (AO), a metachromatic dye that emits different spectra upon binding DNA or RNA (Bernas et al., 2005). We also devised an approach for staining intracellular proteins with fluorescein-5-isothiocyanate (FITC), a fluorochrome that covalently binds with proteins *in vitro* (Jullian et al., 2009). The specificity of RNA staining with AO and of protein staining with FITC was verified in mouse somatic fibroblasts and oocytes by their treatment with RNase A or proteinase K before cell exposure to the dyes. The results showed that, irrespective of the functional status, NLBs of fully-grown mouse oocytes contain RNA and proteins similar to nucleoli fully active in rRNA synthesis. By mild digestion of oocytes with proteinase K, we showed that the nucleolar proteins involved in key steps of ribosome biogenesis (UBF, fibrillarin, NPM1, nucleolin, RPL26) are located not only at the NLB surface, but are also immersed into the NLB mass. Conversely, none of the nuclear proteins examined (SC35, NOBOX, topoisomerase II beta, HP1α, or H3) were detected within NLBs. To determine whether accumulation of the nucleolar proteins is accompanied by accumulation of rRNA, we applied fluorescence *in situ* hybridization (FISH) and oligonucleotide probes targeting 18S and 28S rRNAs. However, the FISH results showed that, in contrast to active nucleoli of growing oocytes and somatic cells (Shishova et al., 2011), the rRNAs are hardly detectable within the NLB mass of GV oocytes. We also failed to reveal transcribed ribosomal genes within NLBs of proteinase K-treated GV oocytes, which was consistent with their absence in NLBs of oocytes examined using BrUTP as a precursor under conventional conditions. Based on our results, we conclude that NLBs of mammalian oocytes serve mainly as storages of nucleolar proteins but not of rRNAs. They may also be impoverished for proteins with nuclear functions.

Materials and methods

Cell culture

NIH/3T3 mouse fibroblasts were purchased from the Russian Cell Culture Collection (Institute of Cytology of the Russian Academy of Sciences, St. Petersburg, Russia) and were free of mycoplasma. The cells were cultured in DMEM (Pan Eco, Russia) containing 10% fetal bovine serum (HyClone, USA), 2 mM L-glutamine, penicillin, and streptomycin (250 units/ml of each) at 37 °C and 5% CO_2.

Animals

Female C57Bl/6 mice were purchased from the Pushchino Nursery of Laboratory Animals (Pushchino, Russia). The animals were kept under pathogen-free conditions with access to tap water and standard chow *ad libitum*. All experiments were performed according to the local law and principles of good laboratory animal care.

Collection of oocytes

Four-to-six-week old females were injected with 7 IU PMSG (pregnant mare's serum gonadotropin) (Sigma-Aldrich, USA) and sacrificed 46–48 h later. Oocytes were collected from ovaries by gentle puncturing of follicles with a needle in M2 medium (Sigma-

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