Overexpression of ribosomal RNA in cumulus cells of patients with polycystic ovary syndrome

Mikhail Polzikov · Sergey Yakovenko ·
Julia Voznesenskaya · Maria Troshina · Olga Zatsepina

Received: 11 March 2012 / Accepted: 26 June 2012 / Published online: 7 July 2012
© Springer Science+Business Media, LLC 2012

Abstract

Objective To evaluate the level of ribosomal RNA (rRNA) in cumulus cells (CCs) from women with polycystic ovary syndrome (PCOS) undergoing ICSI.

Materials and methods The study population included three healthy oocyte donors (control) and three patients with PCOS. RNA expression in CCs was assessed using quantitative real-time PCR assay to measure the pre-rRNA transcripts (5'ETS region), 18 S, 5.8 S and 28 S rRNA.

Results The level of 18 S rRNA is 3.9 times higher ($p=0.036$) and the level of 5.8 S rRNA is 2.9 times higher ($p=0.049$) in CCs of PCOS patients than in CCs of healthy women. The fold change in expression of 28 S rRNA in CCs of PCOS patients also exceeds that in the control group, but did not reach a statistical significance ($p=0.342$).

Conclusion Our observations support the idea that CCs of PCOS patients contain comparatively more ribosomes than CCs of healthy oocyte donors that may indicate a higher proliferation rate or up-regulated translation of protein factors in CCs of PCOS patients.

Capsule We found that cumulus cells of women with polycystic ovary syndrome are enriched in rRNA that may argue in favor of a higher content of ribosomes and, apparently, of up-regulated total protein synthesis.

M. Polzikov · O. Zatsepina
Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry,
Russian Academy of Sciences,
16/10 Miklukho-Maklaya St,
117997 Moscow, Russia

M. Polzikov (✉) · S. Yakovenko · J. Voznesenskaya ·
M. Troshina
“AltraVita” IVF clinic,
4A Nagornaya St,
117186 Moscow, Russia
e-mail: polzikov@yahoo.co.uk

Keywords Cumulus cells · rRNA · In vitro fertilization · Polycystic ovary syndrome

Introduction

It is well known that protein synthesis directly correlates with the synthesis and amount of ribosomes present in a cell [1, 2]. For example, cells capable of proliferation or excreting proteins are more enriched in ribosomes than non-proliferating cells or cells that do not produce proteins for export. A large amount of ribosomes and ribosomal RNA (rRNA) is also accumulated in oocytes during their maturation [3]. The major role in ribosome synthesis is played by the nucleolus, where the three major classes of rRNA, namely 18 S rRNA, 5.8 S rRNA, and 28 S rRNA, are transcribed as a single 47 S pre-rRNA precursor and processed to produce mature cytoplasmic rRNAs [4] (Fig. 1a). In somatic cells, certain amounts of 18 S, 5.8 S, and 28 S rRNA are present in transcriptionally active nucleoli, but the vast majority of mature rRNAs are docked by cytoplasmic ribosomes.

Cumulus cells (CCs) are specialized somatic cells that surround and nourish oocytes by providing a number of specific proteins and enzymes such as GREM1, BDNF, PGK1, CDC42, STAR, and CYP19A1, which are required for oocyte development and fertilization [5]. One could expect, therefore, that disturbances in protein synthesis in cumulus cells may have negative effects on the oocyte quality. However, to the best of our knowledge, ribosome biogenesis has not been studied in CCs of either healthy women or patients with fertility dysfunction. To shed light on this question, we examined the amount of pre-rRNA transcript, 18 S, 5.8 S and 28 S rRNA in the CCs of patients with polycystic ovary syndrome (PCOS), which is a frequent woman infertility disorder that

Fig. 1 **a** A general scheme of the 47 S pre-rRNA transcript in human. 5'ETS—the 5' external transcribed spacer, 18 S—precursor of 18 S ribosomal RNA, ITS1—the first internal transcribed spacer, 5.8 S—precursor of 5.8 S ribosomal RNA, ITS2—the second internal transcribed spacer, 28 S—precursor of 28 S ribosomal RNA, 3'ETS—the 3' external transcribed spacer. Horizontal lines—positions of PCR amplicons for 5'ETS and 18 S, 5.8 S, 28 S rRNA. **b** Graphs representing the fold change in the level of pre-rRNA (5'ETS), 18 S, 5.8 S and 28 S rRNA in CCs of PCOS patients and healthy oocyte donors. The

graphs indicate nearly 4-fold increase of 18 S rRNA and 3-fold increase of 5.8 S rRNA in PCOS patients. The boxes include values between the 25th and 75th percentile of the distribution, while the line within the box represents the median value, dots within the box represent the average values. The whiskers above and below the box indicate the 90th and 10th percentile, respectively, and the asterisks indicate the outliers. PCOS, $n=3$ subjects; healthy donors, $n=3$ subjects; p —probability value

increases the risk for ovarian hyperstimulation syndrome, using quantitative reverse transcription real-time PCR (qRT-PCR).

Materials and methods

Study population

The study population included oocytes of three women with PCOS and of three healthy oocyte donors (the control group). The patients were diagnosed with PCOS on the basis of the ESHRE/ASRM Rotterdam Consensus Meeting criteria (2003) and underwent IVF/ICSI in the “AltraVita” IVF clinic (Moscow, Russia). In the same clinic the healthy donors underwent ovarian stimulation. The women with PCOS had oligomenorrhea or amenorrhea, clinical or biochemical evidence of hyperandrogenism and polycystic ovaries on ultrasound scan with no hyperprolactinemia, thyroid hormone disorders or congenital adrenal hyperplasia. Their clinical, endocrine and ultrasound parameters versus healthy oocyte donors are summarized in Table 1. The controlled ovarian

stimulation was performed using a recombinant FSH (average total dose of 1480 IU for the patients and 2270 IU for the donors) and GnRH antagonist. The retrieval of oocytes was

Table 1 Clinical, endocrine and ultrasound parameters of PCOS patients and healthy oocyte donors (the control group)

Parameters	PCOS patients ($n=3$)	Healthy donors ($n=3$)
Age (yr)	29 (26–35)	24 (23–26)
BMI (kg/m ²)	21 (19–24)	24 (21–26)
FSH (IU/liter)	4,3 (3,1–5,5)	4,5 (4–5,8)
LH (IU/liter)	14,6 (11,2–18,5)	5,3 (4,9–6,3)
Testosterone (nM/liter)	3,8 (2,2–5,7)	1,8 (1,1–2,4)
TSH (mIU/liter)	0,8 (0,2–1,2)	0,9 (0,2–1,1)
Ultrasound parameters		
- Number of follicles	21 (18–25)	6 (4–7)
- Size of follicles (mm)	4,8 (4,2–5,6)	4,5 (4,1–5,1)

n —the number of women in each group, in parentheses the ranges of parameters are indicated

performed after 36 h of hCG administration under ultrasound guidance; the average number of oocytes collected was 27 for the patients and 23 for the donors which were underwent IVF/ICSI procedure. 18 oocytes (MII stage) from one healthy oocyte donor were vitrified. The patients, donors, their oocytes and embryos were not subjected to any procedures which did not satisfy the clinically approved protocols and rules of the Russian Ministry of Health for assisted reproductive technologies (№ 67 by 26/02/2003). The study was approved by the local ethics committee of “AltraVita” IVF clinic. All of the patients signed an official informed consent for participation in the experiment

Cumulus cells collection and total RNA isolation

After collection of the ova, the oocyte-cumulus complexes were immediately separated from the follicular fluid and washed in HTF flushing medium (CooperSurgical, Trumbull, USA). Approximately 5×10^2 CCs were detached from an oocyte with a sharp needle under a dissecting microscope Stemi 2000-C (Carl Zeiss AG, Berlin, Germany) and used for total RNA extraction with a ChargeSwitch RNA Kit (Invitrogen, Paisley, UK). Other CCs were removed by denuding of oocytes in Quinn’s Advantage® Medium with HEPES (CooperSurgical) in presence of 15 IU/ml hyaluronidase (CooperSurgical). The reverse transcription reactions were performed immediately after RNA isolation using AMV-reverse transcriptase, random primers and dNTPs (Fermentas, Chembridge, UK) following the manufacturer’s instructions.

Quantitative reverse transcription real-time PCR (qRT-PCR)

RNA expression was assessed using qRT-PCR assay to assess the amounts of pre-rRNA transcripts (5’ETS region), 18 S, 5.8 S and 28 S rRNA (Fig. 1a). The primers and FAM-conjugated probes for human rRNA (GenBank ID: U13369.1) were designed using AlleleID 7.7 software

(PREMIER Biosoft, Palo Alto, USA) and synthesized by “DNA-Synthesis” (Moscow, Russia). The primer and probe sequences are indicated in Table 2. The positions of the amplicons are shown in Fig. 1a. Human *beta-actin* mRNA (GenBank ID: NM_001101.3) was used to normalize the amount of isolated RNA in each sample. The master mix, qPCRMix-HS (Evrogen, Moscow, Russia), and 2 µl of cDNA were used in a PCR reaction. qRT-PCR was performed three times for each amplicon (i.e., 5’ETS, 18 S, 5.8 S, and 28 S rRNA and *beta-actin* mRNA) using a MiniOpticon RT-PCR system (BioRad, Hercules, USA) with the following parameters: 5 min denaturation at 95 °C and 40 PCR cycles for 15 s at 95 °C, 20 s at 55 °C and 20 s at 72 °C. Melting curve analysis and gel electrophoresis were used to control the specificity and quality of PCR. The data were analyzed using Bio-Rad CFX software (BioRad). Fold-induction values (n) were calculated using the following formula $n = 2^{-\Delta\Delta C_t}$, where C_t represents the mean threshold cycle of all replicate analyses of a given amplicon and ΔC_t represents the difference between the C_t values of the target amplicon and the C_t value of the reference gene (*beta-actin*) [6]. The statistical analysis was performed using Kruskal-Wallis ANOVA on rank using Origin 5.0 software (OriginLab, Northampton, USA), $p < 0.05$ was considered statistically significant.

Results

The major clinical, endocrine and ultrasound parameters of PCOS patients and healthy oocyte donors are summarized in Table 1. One can see that almost all parameters for the both groups are compatible with exception of the levels of LH and testosterone and of the number of ultrasound follicles, which were higher in PCOS patients than in the control group. These observations are in accordance with the ESHRE/ASRM Rotterdam Consensus Meeting criteria for PCOS disorder symptoms [7].

Table 2 Primers and probes used for the amplification of pre-rRNA transcripts (5’ETS region), 18 S, 5.8 S and 28 S rRNA by qRT-PCR as described in [Materials and methods](#)

Amplicon	5'- primers	Probes (FAM-conjugated)	3'- primers
5’ETS (1389–1490 nt)	CACCCTCGGTGAGAAAAG	CTTCTCTAGCGATCTGAGAGGCGTGCC	CTACCATAACGGAGGCAG
18 S rRNA (4840–4976 nt)	GCTGAAACTTAAAGGAATTGA	TCAATCTGTCAATCCTGTCCGTGT	CACGGAATCGAGAAAGAG
5.8 S rRNA (6624–6727 nt)	GACTCTTAGCGGTGGATC	TGTCCTGCAATTCACATTAATTCTCGC	GTTGGAAGTGTGCGATGATC
28 S rRNA (9898–9975 nt)	CCATGGAAGTCGGAATCC	TAACAACTCACCTGCCGAATCAACTA	AGCGCCATCCATTTTCAG
beta-Actin (552–682 nt)	CCTGACTGACTACCTCATG	CTTCTCCTTAATGTCACGCACGATT	CCATCTCTTGCTCGAAGTC

The major nuclear parameters of oocytes after denuding CCs such as the number of mature and immature oocytes, fertilization and blastocyst rates in the both groups of women are summarized in Table 3. They demonstrate that slightly more GV oocytes, but less MI and MII oocytes were obtained from PCOS patients than from healthy oocyte donors, but in the both groups of women oocytes at the MII stage was the dominating type amongst all isolated oocytes. The cytoplasmic maturation stage of MII oocytes before ICSI was similar in the both groups. Under a light microscope (Leica DMI4000, Leica Microsystems GmbH, Berlin, Germany) with a lens $\times 20/\text{NA } 0.4 \text{ Ph2}$ the cytoplasm of oocytes was rather homogenous and devoted of granular aggregates or vacuole clustering. The oocyte fertilization rate was lower in PCOS patients (79 %) than in healthy donors (93 %) (Table 3). The blastocyst rate at the days 5th and 6th after ICSI, which assessed only blastocysts of the excellent and good quality, was also lower in PCOS patients (42 %) than in healthy donors (69 %) (Table 3).

The total amount of 5'ETS, 18 S, 5.8 S, and 28 S rRNA and beta-actin mRNA was analyzed in CCs detached from 102 oocytes underwent ICSI, including 45 oocytes obtained from three PCOS patients and 57 oocytes obtained from three healthy oocyte donors. The levels of pre-rRNA (5' ETS) and 18 S, 5.8 S and 28 S rRNA in CCs of healthy donors was normalized to the level of beta-actin mRNA and revealed equal amounts. As shown in Fig. 1b, the relative content of two classes of rRNAs—18 S and 5.8 S rRNA - is statistically augmented in CCs of PCOS patients in

comparison with that in CCs of healthy donors. Namely, the level of 18 S rRNA is 3.9 times higher ($p=0.036$) and the level of 5.8 S rRNA is 2.9 times higher ($p=0.049$). The fold change in the level of 28 S rRNA was also increased in CCs of PCOS patients, but the difference did not reach a statistically significant value ($p=0.342$). Similar results were also obtained for the 5'ETS region of pre-rRNA ($p=0.648$), which amount is a measure of rDNA transcription rate in nucleoli.

Discussion

PCOS is the most common female endocrinopathy, affecting 5–10 % of women of reproductive age, and it is associated with 75 % of anovulatory infertility disorders [8]. The impediment of ovarian function in PCOS patients can affect both the quality of CCs and oocytes. In this study, we showed that CCs of PCOS patients contain higher amounts of 18 S and 5.8 S rRNA which are synthesized in nucleoli to comprise cytoplasmic ribosomes. This finding argues in favor of a higher content of ribosomes and, apparently, of an up-regulated level of total protein synthesis in CCs of women with PCOS.

Ribosome biogenesis is an evolutionarily conserved process. In eukaryotes, it starts in the nucleolus with transcription of the common pre-rRNA precursor for 18 S, 5.8 S, and 28 S rRNAs (Fig. 1a) and ends in the cytoplasm with the formation of mature ribosomes. In the nucleolus, pre-rRNA forms stable links with proteins of cytoplasmic ribosomes and transient links with proteins involved in rRNA maturation [4]. rRNA transcription and ribosome biogenesis are controlled by a number of cellular pathways such as the PI3 kinase/mTOR, MYC and RAS/ERK pathways, whereas augmented rRNA synthesis is necessarily for cell proliferation and growth [2, 9, 10].

Therefore, it is tempting to speculate that higher amounts of rRNA present in CCs of PCOS patients are resulted from a higher proliferation rate of the cells. This assumption is in a good concordance with recent findings which demonstrate increased proliferation and decreased apoptotic rates in granulosa cells of PCOS patients [11].

Alternatively, higher levels of 18 S and 5.8 S rRNA in CCs of PCOS patients may be associated with an elevated level of proteins or enzymatic factor in CCs. As far as the complex signaling between CCs and oocytes is bi-directional via the gap junctions formed by CCs and *zona pellucida* of oocytes [12], one could suppose that activation of ribosomal gene expression in CCs of woman with PCOS may lead to disturbances in oocyte-cumulus communications and development of embryos after ICSI. In a line with this assumption, we observed differences in the number of mature MII oocytes, fertilization and blastocyst rates

Table 3 The total number of oocytes at different nuclear maturation stages before ICSI procedure and the total number of fertilized oocytes, unfertilized oocytes and blastocysts after ICSI in PCOS patients and healthy oocyte donors

Stage	PCOS patients (<i>n</i> =3)	Healthy donors (<i>n</i> =3)
GV Prophase I and GVBD oocytes	9	4
MI oocytes	3	7
MI oocytes	33*	46 [#]
Fertilized oocytes (i.e., zygotes with 2PN)	26	26
Unfertilized oocytes (i.e., zygotes with 0PN)	7	2
Fertilization rate	79 %	93 %
Blastocysts of the excellent and good quality at the days 5th and 6th after ICSI	11	18
Blastocyst rate	42 %	69 %

n—the number of women in each group

*all MII oocytes obtained were used for ICSI procedure

[#]oocytes obtained from one healthy oocyte donor (18) were vitrified

between PCOS patients and healthy oocyte donors, which were lower in the patients (Table 3). Thus, the level of rRNA in CCs of PCOS patients reversibly correlates with the outcome of mature oocytes and embryos. However, we can not completely exclude that the higher level of rRNA in CCs of PCOS patients may result from specific (i.e., different from the healthy oocyte donors) reaction of PCOS patients to ovarian stimulation. Nowadays, data on rRNA amount in CCs of patients with other reproductive disorders, which could contribute to discussion of this admission, are absent. To shed more light on a link between a higher amount of rRNA in CCs and oocyte developmental capacities in PCOS patients, further studies should be undertaken.

Acknowledgments The study was financed by the Russian Ministry of Education and Science (grants 14.740.11.0925 and 14.740.11.0121).

Conflict of interest None

References

1. Boulon S, Westman BJ, Hutten S, Boisvert FM, Lamond AI. The nucleolus under stress. *Mol Cell*. 2010;40:216–27.
2. Pederson T. The nucleolus. *Cold Spring Harb Perspect Biol*. 2011;3.
3. Wolgemuth DJ, Jagiello GM, Henderson AS. Quantitation of ribosomal RNA genes in fetal human oocyte nuclei using rRNA: DNA hybridization in situ. Evidence for increased multiplicity. *Exp Cell Res*. 1979;118:181–90.
4. Boisvert FM, van Koningsbruggen S, Navascues J, Lamond AI. The multifunctional nucleolus. *Nat Rev Mol Cell Biol*. 2007;8:574–85.
5. Assou S, Haouzi D, De Vos J, Hamamah S. Human cumulus cells as biomarkers for embryo and pregnancy outcomes. *Mol Hum Reprod*. 2010;16:531–8.
6. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) method. *Methods*. 2001;25:402–8.
7. Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). *Hum Reprod*. 2004;19:41–7.
8. McVeigh E, Homburg R, Guillebaud J. Polycystic ovary syndrome. In: *Oxford Handbook of Reproductive Medicine and Family Planning*. Oxford University Press; 2008:57–71.
9. Derenzini M, Trere D, Pession A, Montanaro L, Sirri V, Ochs RL. Nucleolar function and size in cancer cells. *Am J Pathol*. 1998;152:1291–7.
10. McStay B, Grummt I. The epigenetics of rRNA genes: from molecular to chromosome biology. *Annu Rev Cell Dev Biol*. 2008;24:131–57.
11. Das M, Djahanbakhch O, Hacıhanefioglu B, Saridogan E, Ikram M, Ghali L, Raveendran M, Storey A. Granulosa cell survival and proliferation are altered in polycystic ovary syndrome. *J Clin Endocrinol Metab*. 2008;93:881–7.
12. Huang Z, Wells D. The human oocyte and cumulus cells relationship: new insights from the cumulus cell transcriptome. *Mol Hum Reprod*. 2010;16:715–25.