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Short communication

28S rRNA is inducibly pseudouridylated by the mTOR pathway translational control in CHO cell cultures

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ABSTRACT

The mTOR pathway is a conserved master regulator of translational activity that influences the fate of industrially relevant CHO cell cultures, yet its molecular mechanisms remain unclear. Interestingly, rapamycin specific inhibition of the mTOR pathway in CHO cells was found to down-regulate the small nucleolar RNA U19 (snoRNA U19) by 2-fold via translatome profiling. snoRNA U19 guides the two most conserved pseudouridylation modifications on 28S ribosomal RNA (rRNA) that are important for the biogenesis and proper function of ribosomes. In order to further understand the role of snoRNA U19 as a potential player in the mTOR pathway, we measured 28S rRNA pseudouridylation upon rapamycin treatments and/or snoRNA U19 overexpression conditions, thereby characterizing the subsequent effects on ribosome efficiency and global translation by polysome profiling. We showed that 28S rRNA pseudouridylation was increased by rapamycin treatment and/or overexpression of snoRNA U19, but only the latter condition improved ribosome efficiency toward higher global translation, thus implying that the mTOR pathway induces pseudouridylation at different sites along the 28S rRNA possibly with either positive or negative effects on the cellular phenotype. This discovery of snoRNA U19 as a new downstream effector of the mTOR pathway suggests that cell engineering of snoRNAs can be used to regulate translation and improve cellular growth in CHO cell cultures in the future.

1. Introduction

Translational control via the mTOR pathway plays important role in regulating the growth and productivity of the industrially relevant CHO cells producing monoclonal antibodies (Courtes et al., 2013a; Dreesen and Fussenegger, 2011); however, the underlying cellular mechanisms remain elusive. Experimentally, targeted inhibition of the mTOR pathway by rapamycin treatment in CHO cells down-regulated a number of genes, as such leading to reduced cellular growth (Courtes et al., 2013b). Interestingly, a recent study reported that growth arrest upon rapamycin treatment in human T-lymphocytes involves changes in expression of the small nucleolar RNA host gene (SNHG) GAS5 (Williams et al., 2011), which motivated us to explore the emerging role of small nucleolar RNA (snoRNAs) in the mTOR pathway regulation of CHO cells.

In mammalian cells, SNHGs belong to the family of 5′TOP mRNA (Smith and Steitz, 1998) whose expression is regulated by the mTOR pathway (Meyuhas, 2000), and they encode for snoRNAs in their intron regions (Ganot et al., 1997). snoRNAs are a group of non-coding RNAs responsible for posttranscriptional modification of ribosomal RNAs (rRNA) such as pseudouridylation, the most common and evolutionary conserved modification from yeast to human cells (Charette and Gray, 2000). Insertion of pseudouridines generates more hydrogen bond donors in the rRNA structure, thus providing the potential to form new intra- and inter-molecular interactions between partner proteins and mature ribosomes (Jack et al., 2011). Although the exact effect and function of pseudouridine modifications remain unclear, it is suggested that these modifications may serve to adjust rRNA structure for regulating the accuracy and efficiency as well as the biosynthesis of ribosomes (Piekna-Przybylska et al., 2008). In yeast, combinatorial depletions of five snoRNAs altered the structure of the large ribosomal subunit, which led to a shift of ribosome from polysome toward monosomes

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resulting in a 45% decrease of global translation output (King et al., 2003). Similarly, a substitution mutation of four snoRNAs guiding pseudouridylation modification in the loop and stem structures of the core H69 region known to interact with tRNAs (Yusupov et al., 2001), reduced amino acid incorporation rates in vivo and increased stop-codon readthrough activity (Liang et al., 2007). A mechanistic study on the same pseudouridylation sites of the H69 region further demonstrated that the lack of pseudouridylated residues led to conformational change of rRNA in the large subunit so as to assure correct positioning of critical rRNA bases involved in tRNAs accommodation (Baxter-Roshek et al., 2007). Interestingly, thermodynamic and NMR based studies revealed that pseudouridylated residues can stabilize or destabilize an RNA hairpin structure when located at a stem loop junction or in single-stranded loop region respectively (Meroueh et al., 2000). Alternatively, defect in pseudouridylation was also observed to strongly delay rRNA processing thus modulating ribosome synthesis (Liang et al., 2009). Indeed, a number of individual snoRNAs guiding pseudouridylation at specific sites within core and highly conserved regions of rRNA, have proven to affect cellular growth phenotype in various organisms. For example, knockdown of snoRNA U19, the snoRNA that guides two most conserved pseudouridylation in 28S rRNA from yeast to human (Badis et al., 2003), induced a growth inhibition in yeast as compared to the wild type. Moreover, overexpression of snoRNA-42 in bronchial epitheliums cells, a snoRNA found in high levels in lung tumors, increased cell growth and colony formation (Mei et al., 2012).

In this study, translatome analysis of CHO cells upon mTOR pathway inhibition by rapamycin allowed us to identify the non-protein-coding SNHG4 hosting the equivalent of human snoRNA U19 as potential downstream effector in this signaling pathway. Thus, deciphering snoRNA U19 implication in gene expression regulation of CHO cell, in line with translational control of 5′TOP mRNAs through mTOR pathway is of great interest to understand new molecular mechanisms that contribute to regulating cellular growth. Toward this end, we overexpressed snoRNA U19 for characterization of its potential role in the mTOR pathway and its effects on pseudouridylation, ribosome efficiency and cellular growth phenotype. These preliminary evidences reveal that snoRNAs, alike other short non-coding RNAs such as miRNA, hold promising potential for improving CHO cell cultures.

2. Materials and methods

2.1. Cell culture and rapamycin treatment

CHO-DG44 cell line was cultivated in a protein free and chemically defined property medium in shake flasks. All cell cultures were performed essentially as described in our previous work (Courtes et al., 2013a). For rapamycin treatment, 20 ng mL^{-1} was added on day 0 of cultures.

2.2. Translatome analysis

Translatome analysis was conducted by quantifying the ratio of rapamycin translatome over control translatome for each gene. Translatome data for the control condition were previously generated and validated by Courtes et al. (2013a), and translatome data under rapamycin treatment were similarly calculated as the ratio of polysome to monosome enriched pools based on the translatomic platform for CHO cell cultures. In brief, total RNA was extracted from cells and separated on 10–50% sucrose gradient. RNAs from 13 fractions were pooled together in pool A (polysome enriched) and pool B (monosome enriched) and purified via phenol–chloroform (Sigma–Aldrich) extraction before quantification on microarrays.

2.3. Overexpression of snoRNA U19

The insert containing snoRNA U19 was amplified from total CHO cells gDNA template with the forward (GGCGCTAGCTAACTTACAATCAGGCAAGTG) and reverse (GGCGGATCCCCTGATGGAGTCAGTTTTCTC) primers and was inserted into vector pcDNA^{TM}3.1/Hygro (Invitrogen). CHO cells were transfected using the Amaxa® Cell Line Nucleofector® Kit V (Lonza) according to the manufacturer instruction. Stable pools were selected with 300 μg mL^{-1} hygromycin (Clontech) 24 h post-transfection.

2.4. Quantification of snoRNA U19

Total RNA was extracted from 5×10^6 cells with Trizol (Invitrogen) as per the manufacturer's instructions. Equal volumes of total RNA samples were subjected to gDNA digestion with the RQ1 RNase-Free DNase (Promega) and were supplemented with 10 μL of the internal control gene *thrB* of *Bacillus subtilis* (dilution 1:2000; Affymetrix). RNA samples were then polyadenylated by polyA polymerase (Ambion) according to the method developed by Ro et al. (2006). Equal volumes of polyadenylated RNA samples per day were subsequently reversed transcribed using the ImProm-II^{TM} Reverse Transcription System (Promega). Finally, snoRNA U19 was quantified via qRT-PCR using the supermix SsoFast^{TM}EvaGreen® (Biorad), for 40 cycles at 95 °C for 30 s and 60 °C for 10 s in iQ5 cycler system (Biorad). Measured CT values were processed according to the standard Δ(CT) method (Livak and Schmittgen, 2001) with the internal control *thrB* gene in order to account for possible loss of RNA during the various extraction and purification steps.

2.5. Quantification of pseudouridine in 28S rRNA

Pseudouridine was quantified in 28S rRNA in triplicate from mid-exponential growth phase for each experimental condition. 28S rRNA was purified from total RNA by size-exclusion chromatography as described in Chionh et al. (2013). Following concentration on a 10 kD filter (Pall, YM-10), the RNA was hydrolyzed and dephosphorylated into ribonucleoside form (Chan et al., 2010) and proteins were removed by a second 10 kD filtration. The ribonucleosides were then resolved by HPLC (Thermo Scientific Hypercarb reversed-phase column, 100 mm × 2.1 mm, 3 μm particle size) with a gradient of acetonitrile in water containing 0.1% formic acid at a flow rate of 0.2 mL min^{-1} at 65 °C: 0–5 min, 5%; 15–25 min, 5–28%; 25–75 min, 28–75%; 25–30 min, 75%. Synthetic pseudouridine (Berry & Associates) eluted at 12.7 min under these conditions. The HPLC system was directly coupled to an Agilent 6410 QqQ LC/MS mass spectrometer with an ESI source operating in positive ion mode: gas temperature, 350 °C; N_2 gas flow, 10 L/min; nebulizer pressure, 20 psi; and capillary voltage, 3500 V. Pseudouridine was detected by setting Q1 to transmit the parent ion with m/z 125 (unit resolution) and Q3 set to monitor the m/z = 191 and 125 product ions resulting from collision-induced dissociation (collision energy, 10 eV; fragmentor voltage, 80 V; dwell time 200 ms), as described previously (Chan et al., 2010; Dudley et al., 2005). An example of the LC–MS/MS chromatogram for these CID fragmentation products is shown in Fig. S1A. To account for sample variation, the signal for pseudouridine was normalized by dividing the integrated MS peak for m/z 245 → 191 by the integrated 260 nm absorbance peak for cytidine measured by an in-line UV detector.