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SMALL NUCLEOLAR AND SMALL CAJAL BODY-SPECIFIC RNAs EXPRESSION PROFILE OF OXIDATIVE STRESSED AND NORMAL RPE CELLS SUGGESTS UNKNOWN REGULATIVE ASPECTS OF RETINITIS PIGMENTOSA ETIOPATHOGENESIS

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ABSTRACT. Recent discoveries on non coding RNAs (ncRNAs) suggest that a huge number of regulative mechanisms of transcription and translation in eukaryotes could represent the key to improve our knowledge on many diseases etiopathogenesis, like Retinitis pigmentosa (RP). Among ncRNAs, small nucleolar RNAs (snoRNAs), and their sub - group Small Cajal Body - specific RNAs (scaRNAs), represent an undiscovered world. We compared snoRNAs expression changes, coming from whole transcriptomes analyses, between two group of Retinal Pigment Epithelial (RPE) cells, treated with the oxidant agent oxidized low-density lipoprotein (oxLDL) and untreated, respectively, considering four time points (1h, 2h, 4h, 6h). We found 84 snoRNAs, clustered in five groups based on showed altered expressions in treated samples and targeting several ribosomal RNAs (rRNAs), presented their host genes involved in several biochemical pathways. One of them, involving the "silencing activity mediated by the interferon-γ-activated inhibitor of translation (GAIT) translational control system", seems to be very interesting and only speculated to be associated to RP. SnoRNA expression analysis of oxidative stress induced RPE cells suggested that ncRNAs could play a relevant role in RP etiopathogenesis, regulating pathways directly or indirectly related to the considered disease.

1. Introduction

Recent discoveries on non coding RNAs (ncRNAs) suggest that a huge number of regulative mechanisms of transcription, maturation (e. g. splicing, (Scimone *et al.* 2017) and translation in eukaryotes could represent the key to improve our knowledge on many diseases etiopathogenesis (Santosh *et al.* 2015). In eukaryotes, the ribosomal RNA (rRNA) requires several site-specific post-transcriptional nucleotide modifications, most relevant of which are represented by the addition of a methyl group to the 2'-hydroxyl group of a ribose residue (2'-O-methylation) and the isomerization of uridine to pseudouridine (Ψ), a reaction known as pseudouridylation (Arai *et al.* 2013). Both processes occur in the nucleolus and involve a huge number of small nucleolar RNAs (snoRNAs), made of 60-300 nucleotides, as well as multicomponent complexes, constituted by small nucleolar ribonucleoprotein

(snoRNP) (McMahon *et al.* 2015). SnoRNAs can be divided into two classes which possess distinctive sequence elements: the highly conserved box C/D snoRNAs promote the 2'-O-methylation of specific sites on rRNA, thanks to the methyltransferase fibrillarin (Scott *et al.* 2012). The second group, represented by the box H/ACA snoRNAs, guide pseudouridine modifications at specific sites on rRNA, with the help of the pseudouridine synthase dyskeri and several additional proteins like GAR1 and NOP10 (Watkins and Bohnsack 2012). Interestingly, there is a growing evidence that guide snoRNA targets are not limited to rRNA, as evidenced by identification of many "orphan snoRNAs" with already unknown target RNAs (Dupuis-Sandoval *et al.* 2015). Even if the exact function of different types of rRNA modifications is not totally clear, there is now a mounting hypothesis that the machinery needed for site-specific rRNA modifications is required for normal development and is altered in numerous human pathologies, as cancer, neurodegenerative and genetic diseases (Stepanov *et al.* 2015). Retinitis pigmentosa is a genetic disease characterized by retinal degeneration and followed by a slow and progressive death of photoreceptors and retinal pigment epithelium (RPE) cells, leading to impairment of visual neurotransmission (Natarajan 2013). The term "pigmentosa" deals with the typical appearance, during the advanced states of the disease, of abnormal areas of pigment into the back portion of the eye (Zhang 2016). Many evidences support the role of snoRNAs in normal retinal development and functions, but exact mechanisms are still unknown (Liu *et al.* 2016). Moreover, snoRNAs expression data come only from the analysis of murine small non coding RNAs but, due to structural and functional differences between human and mouse retinas, they are not totally useful (Soundara Pandi *et al.* 2013). Therefore, an improved knowledge of human retina snoRNAs, especially of patients affected by retinal disease, could lead to better understanding the unappreciated functional role of snoRNAs related to physiopathology of this tissue. In our work, we studied the complexity of human retina snoRNAs, analyzing data from human retinal pigment epithelium (RPE) cells transcriptomes. RPE is made of a single layer of cells playing a key role in maintenance, regulation and protection of the photoreceptor layer (Strauss 1995). Due to its specific proteins, RPE regulates the trafficking of nutrients and waste products to and from the retina, help to renew outer segments by phagocytizing the spent discs of photoreceptor outer segments, protects the outer retina from excessive high-energy light and light-generated oxygen reactive species (ROS) and maintains retinal homeostasis through the release of diffusible factors (Amram *et al.* 2017). In details, we compared snoRNAs expression changes between two group of RPE cells, treated with the oxidant agent oxidized low-density lipoprotein (oxLDL) and untreated, respectively, considering four time points (1h, 2h, 4h, 6h) over basal one (time zero). oxLDL was selected due to its known involvement in neurodegenerative diseases pathogenesis but, especially, because it is well known that high cholesterol level could be linked to RP development (Rodriguez and Larrayoz 2010). Main purpose of our work was to discover which snoRNAs changed during oxidative stress induction and which are their targets, in order to better understand how ROS might lead to RP development.

2. Materials and Methods

This study was approved by the Ethics Committee of Azienda Policlinico Universitario "G. Martino" Messina.

2.1. Cell culture. Human RPE-derived Cells (H-RPE - Human Retinal Pigment Epithelial Cells, Clonetics™, Lonza) were maintained at 1x10^6 $\frac{cells}{ml}$ culture in RtEGM™ Retinal Pigment Epithelial Cell Growth Medium BulletKit® (Clonetics™, Lonza) with 2% FBS, 100 $\frac{units}{ml}$ of penicillin and 100 $\frac{\mu g}{ml}$ of streptomycin and incubated at 37 $^\circ$ C with 5% CO_2. Finally, 100 $\frac{\mu g}{ml}$ of oxLDL was added to the treated group, after 24 hours.

2.2. Total RNA sequencing. RNA was extracted by TRIzol™ Reagent (Invitrogen™, ThermoFisher Scientific), following manufacturer's protocol, and quantified at Qubit 2.0 fluorimeter by Qubit® RNA assay kit (Invitrogen, Life Technologies). Expression analysis compared the transcriptome of Human RPE cells treated with 100 $\frac{\mu g}{ml}$ of oxLDL with untreated ones, both at time zero and at four following different time points (1h, 2h, 4h, 6h). Libraries were generated using $1\mu g$ of total RNA and the Ion Total RNA-Seq Kit v2 (ThermoFisher Scientific), then purified by Dynabeads® Magnetic Beads and quantified at Qubit® 2.0 fluorimeter with Qubit® dsDNA HS Assay Kit. Specific libraries amount was used for clonal amplification realized with Ion PI™ Template OT2 200 Kit v2 (ThermoFisher Scientific) on Ion One Touch™ 2 System; template-positive Ion Sphere Particles were enriched at Ion One Touch™ Enrichment System. Sequencing runs were performed on an Ion Proton™ Sequencer (Ion Torrent technology, ThermoFisher Scientific), using the Ion PI™ Sequencing 200 Kit v2 and the Ion PI™ Chip Kit v2 (ThermoFisher Scientific). The experiment was thrice repeated.

2.3. Quality data validation and real mapping. Sequence reads were generated from RPE specific cDNA libraries on the Ion Torrent Proton. Low quality, ambiguous and containing adaptor reads (average per base Phred score < 28) were removed from obtained raw data. The quality of analyzed data was checked using the FastQC (v.0.11.5) and QualiMap (v.2.2.1) software. The filtered data was, then, analyzed by CLC Genomics Workbench v.10.1.1 (https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/) using *Homo sapiens* genome hg19 and Ensembl RNA database v.74 as references. RNA - Seq analysis was conducted using the following settings: quality trim limit = 0.02, ambiguity trim maximum value=2. Map to annotated reference: minimum length fraction and minimum similarity fraction=0.7, maximum number of hits/read=2, type of organism = eukaryote, paired settings = default.

2.4. Small RNA Analysis. The applied approach evaluated the different types of small RNAs in the data and compared them to selected databases of small RNAs. Once imported whole RNA - Seq data, the small RNAs were extracted and counted, in order to create a small RNA sample that could be used for further steps. Sequences were filtered basing on length (reads below 60 bp and above 300 bp were discarded) and on minimum sampling count (set at 1). Subsequently, the number of reads mapping on each snoRNA was counted and, then, normalized using either the Trimmed Mean of M-values (TMM) method (Robinson and Oshlack 2010) or reads per million (CPM). Finally, the snoRNA sample produced when counting the tags was, then, enriched by comparing the tag sequences with the annotation resources Ensembl non -coding RNA database (v.74) (Zerbino *et al.* 2016), Genecode Release 27 (Harrow *et al.* 2012) and Database of small human noncoding RNAs (DASHR) (Leung *et al.* 2016).

2.5. Gene expression and statistical analysis. The original expression values were log2 transformed and normalized in order to ensure that samples are comparable and assumptions on the data for analysis are met (Feng *et al.* 2014). In order to highlight the snoRNAs with different expression levels between untreated and treated samples, and during four analyzed time points, we classified them into two groups, based on count ratios (fold - change): 1) Up - regulated (fold - change >1); 2) Down - regulated (0< fold - change <1). Moreover, due to fold - changes linearity, we chose to replace any value smaller than 1 (i. e. for downregulation) with its negative reciprocal one, in order to make the variation more noticeable (for instance, 2-fold downregulation is indicated by a value of -2 instead of 0.5). Due to a limited availability of biological replicates for each of the experimental group studied (only 3 replicates for each selected time point), but with many features to be analyzed simultaneously (snoRNAs in a whole transcriptome), we applied the Empirical analysis of DGE (EDGE) statistical algorithm, which implements the "Exact Test" for two-group comparisons developed by Robinson and Smyth (Robinson and Smyth 2008). The test assumes that the count data follows a Negative Binomial distribution, and not the Poisson one, allowing for a non-constant mean-variance relationship. The "Exact Test" of Robinson and Smyth is similar to Fisher's Exact Test, but also accounts for overdispersion caused by biological variability. The snoRNAs uniquely identified in the RPE cells with at least 6 unique gene reads, greater than one-fold (up-regulated) or lower than one-fold (down-regulated) changes in expression based on expression values ratio, and with Bonferroni - adjusted p-values lower than 0.05, were selected for functional categorization of differentially expressed snoRNAs.

2.6. snoRNA target identification and host genes pathway analysis. Obtained snoRNAs were firstly analyzed by the experimentally validated snoRNAs database snoRNABase (Lestrade and Weber 2006), and then investigated by snoRNA Orthological Gene Database snOPY (Yoshihama *et al.* 2013) web - based archive and by Ingenuity Target Explorer (QIAGEN, Inc., https://targetexplorer.ingenuity.com/), in order to define specific targets and host genes. Once obtained them, a pathway analysis of selected host genes was performed by Cytoscape and its plug - in ClueGO (Bindea *et al.* 2009).

3. Results

3.1. Sequencing analysis and mapping statistics. RNA sequencing carried out on Ion Torrent yielded a total of 11,214,300 quality reads (mean mapping quality=32,92) with mean read length of 155.03 bp. All reads were previously aligned to GRCh37/hg19 reference assembly, and then to known small human noncoding RNAs (DASHR) and GRCh37 non - coding RNAs (ncRNAs). About 71,500 Small RNAs were founded in all samples, 69,158 of which annotated and about 2,341 unannotated. In details, 84 snoRNAs, resulted from mapping, showed expression alterations in analyzed time points (see Table 1 in Supplementary Files, separately available on the article's web page). All previous mapping statistics are based on average values calculated for all three replicates in each time point.

3.2. Expression analysis. The mean length of resulted snoRNAs was 129.6 bp for snoRNAs, with an average value of 354 bp for scaRNAs. We identified a total of 84 snoRNAs

with an average expression level of 23.615 reads across all considered treated and untreated RPE cells cultures. The variability was significant across samples, with an expected lower trend for expressed scaRNAs. There is a specific fold - change range during analyzed time points, with the highest value of 9.583 reached by SNORD55 and the lowest of -8.361 expressed by SNORD110. Interestingly, we found seven clusters of snoRNAs showing interesting trends through analyzed time points: *Cluster 1*, showing the highest number (26) of deregulated snoRNAs, evidenced a global increase of expression in treated samples (average fold - change = 2.169). *Cluster 2*, made of 17 snoRNAs, evidenced a huge decrease (average fold - change = -0.915) in treated samples through all selected time points. *Cluster 3*, represented by 14 snoRNAs, and *Cluster 4*, made of 12 elements, highlighted only one variation during a single time point, with a positive value of fold - change (average fold - change = 0.768) and a negative one (average fold - change = -0.603), respectively. *Cluster 5* and *Cluster 6*, made of 5 snoRNAs each other, showed a curious trend similar to the last two previously described ones, but with two down - regulations (fold - change = -1.949) or two up - regulations (average fold - change = 1.539) through all selected time points. Finally, *Cluster 7*, made of 4 snoRNAs, resulted up - regulated in 50% of time points and down - regulated in the other 50% (average fold - change = 0.084). Only SNORD69 showed no expression changes during the experiment. Detailed expression data are available in Figure 1 and in Table 2 of Supplementary Files (separately available on the article's web page).

3.3. snoRNAs target identification and host genes pathway analysis. Sixty-eight different residues of ribosomal RNAs resulted as targets of 84 found altered snoRNAs in snoRNABase, snOPY and Ingenuity Target Explorer. Specific targets of 5 H/ACA box snoRNAs (SNORA18, SNORA53, SNORA59A, SNORA59B, SNORA11D) and of 10 C/D box snoRNAs (SNORD23, SNORD89, SNORD109B, SNORD116-24, SNORD116-6, SNORD116-8, SNORD22, SNORD83A, SNORD83B, SNORD84) resulted, instead, still unknown. Detailed report of all analyzed snoRNAs targets is available in Table 2. Afterwards, 6 "macro - pathways" ("L-13-mediated translational silencing of Ceruloplasmin expression", "Cytoplasmic translation", "RNA helicase activity", "Dissociation of L13a from the 60S ribosomal subunit", "eIF3 and eIF1A bind to the 40S subunit" and "Association of phosphor-L13a with GAIT element of Ceruloplasmin mRNA") (Figure 2) and 125 "sub - pathways" resulted from host genes pathway analysis (see Table 3 in Supplementary Files, separately available on the article's web page).

4. Discussion

In the wide world of genetic pathologies, little is known about regulative non - coding RNAs involvement (D'Angelo *et al.* 2012; Adams *et al.* 2017). The only validated knowledge regards miRNA role (D'Angelo *et al.* 2013), which opened new frontiers about 3' UTR and intron variants involvement into etiopathogenesis of such pathologies (Scimone *et al.* 2015), giving ncRNAs the status of most promising targets of experimental therapies (Guzman-Aranguez *et al.* 2013). Among them, Retinitis pigmentosa, an ocular disease with very heterogeneous phenotypes, shows unusually complex molecular genetic causes, most of which still unknown (D'Angelo *et al.* 2017). Using deep sequencing technologies, we analyzed the whole transcriptome of RPE cells, exposed to ox-LDL, during a follow-up of four time points (1h, 2h, 4h and 6h) after exposure, and compared to untreated ones. Thanks

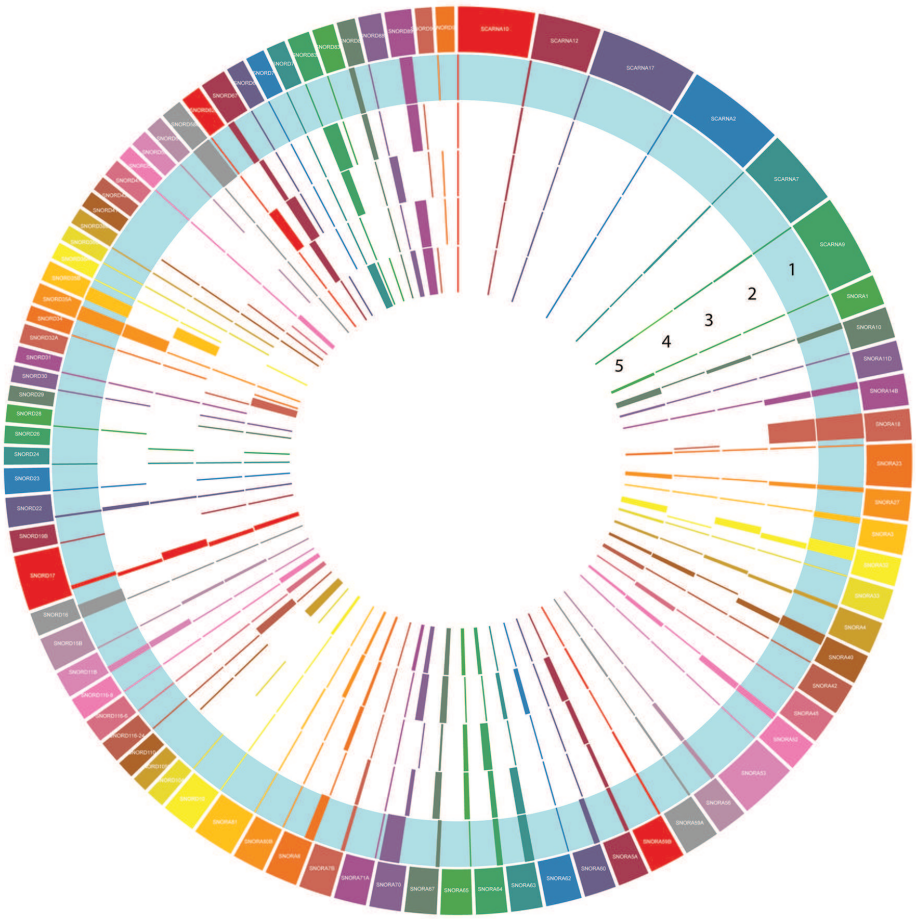

FIGURE 1. Circular plot made by CIRCOS (http://circos.ca) highlighting fold – changes of all analyzed snoRNA during selected time points in each concentric circle. Larger width means greater fold – change. Fold – change 1. 0h vs 1h – Treated. 2. 1h (Treated vs Untreated). 3. 2h (Treated vs Untreated). 4. 4h (Treated vs Untreated). 5. 6h (Treated vs Untreated).

to the high coverage of our sequencing experiment, along with parallel analysis of three replicates for each selected group for each time point, we obtained reliable data, overcoming possible bias - related variability in snoRNA expression levels and nucleotide sequences. Oxidative stress plays a critical role in the etiopathogenesis of RP (Campochiaro *et al.* 2015), especially targeting RPE cells, very sensible because of high metabolic demand, needed for processes like physiological phagocytosis and life-long light illumination. Impairment of such functions could lead to pathobiological modifications like outer blood-retinal barrier (BRB) dysfunctions, alterations of extracellular matrix (ECM) components, inhibition

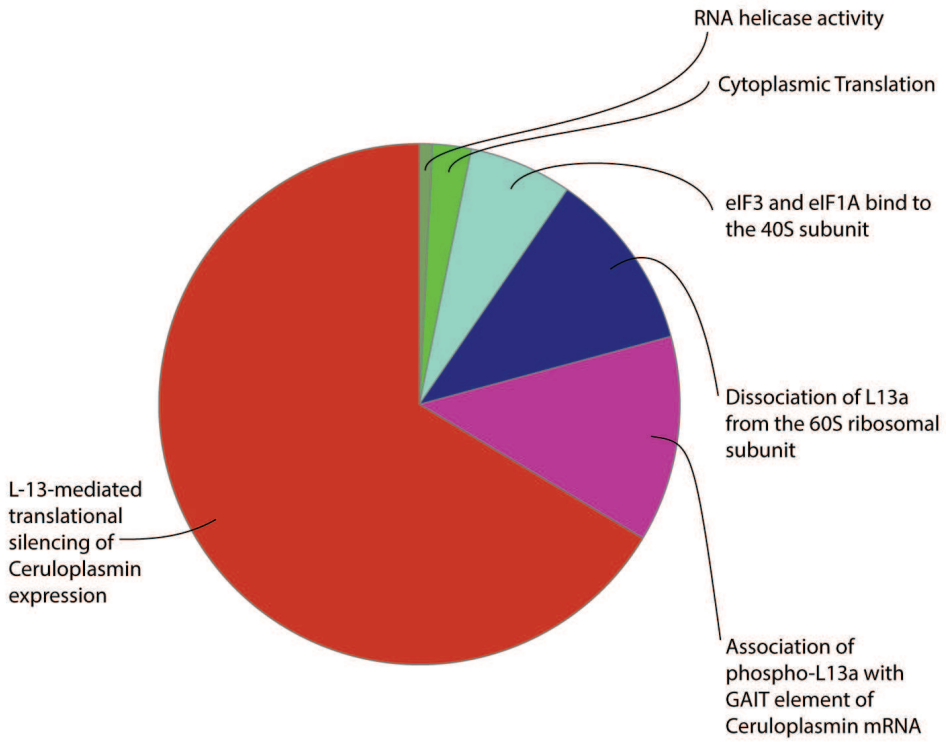

FIGURE 2. ClueGO pathway analysis of snoRNAs host genes from snoRNABase and snOPY. ClueGO analysis highlighted a rich and very clustered network of possible involved pathways for both snoRNABase and snOPY host genes for selected snoRNAs. Details are available in Table 3.

of photoreceptors outer segments processing, increasing of RPE cells senescence and/or apoptosis (Mitter *et al.* 2014). Moreover, several snoRNAs were already detected in murine retina, suggesting a possible involvement in retinal development and activity (Liu *et al.* 2016). Although how such functions could be explicated need to be yet totally understood, several evidences highlight snoRNAs role in influencing translational fidelity, stop codon recognition and ribosome - ligand interactions (Wilusz and Wilusz 2014). Additionally, snoRNAs could play a fundamental role in chromatin remodeling, in miRNA - like post - transcriptional gene silencing and in lncRNA - like RNA splicing modulation (McMahon *et al.* 2015). Intriguingly, the most interesting snoRNAs research area regards the ability for pseudouridine in nonsense codon suppression, potentially altering the coding potential of non-canonical RNA substrates, such as mRNAs (Weischenfeldt *et al.* 2005). For this purpose, we realized a pathway analysis of snoRNAs host genes to highlight possible involvement in Retinitis pigmentosa pathology. Host gene pathway analysis evidenced that alteration of found snoRNAs expression could impair the interferon-γ-activated inhibitor of translation (GAIT) translational control system, made of glutamyl-prolyl tRNA

synthetase (EPRS), NS1-associated protein 1 (NSAP1), ribosomal protein L13a (L13a), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mukhopadhyay *et al.* 2009). It controls the transcript - selective translation of functionally related genes, binding structural GAIT elements in the 3'-untranslated regions (UTRs) of multiple inflammation - related mRNAs, including ceruloplasmin (Cp), and represses their translation (Fox 2015). Ceruloplasmin is a multifunctional, copper-binding α -globulin, with antioxidant capabilities, due to the discharge of electrons, e.g. during conversion of Fe(II) to Fe(III) (Eid *et al.* 2014). In our analysis, we have seen interestingly changes in pathways involving L13a, an integral constituent of the ribosome 60S subunit (Eid *et al.* 2014). In details, it was highlighted a possible alteration in dissociation of L13a from the 60S ribosomal subunit, following DAPK-ZIPK kinase axis phosphorylation, and an association of phospho-L13a with GAIT element of ceruloplasmin, with the possible block of eIF3-containing 43S pre-initiation complex, thereby promoting the L-13a-mediated translational silencing of ceruloplasmin expression. *RPL13A* mRNA is co - transcribed with four box C/D snoRNA genes, U32a, U33, U34 and U35a, located within four *RPL13A* introns (Michel *et al.* 2011), and globally down - expressed in our data. Such results could be compatible with alterations in ceruloplasmin levels, influencing both the transport of copper and the functioning of RPE (Hadziahmetovic *et al.* 2008), as seen in Indian patients of RP (Gahlot *et al.* 1976) and macular degeneration (Newsome *et al.* 1986). Additionally, alterations of copper metabolism could impair allosteric effects on reducing misfolded aggregates, as in other neurodegenerative disorders including Alzheimer disease (Pal *et al.* 2014). Moreover, DAPK and ZIPK are validated regulators of programmed cell death pathways, suggesting the possibility that GAIT - mediated translational repression of the kinases could contribute to regulation of apoptosis. Additionally, it was seen that GAIT complex assembly is inhibited by ox - LDL, resulting in sustained production and accumulation of GAIT system target proteins like Cp (Arif *et al.* 2017). Moreover, deletion of the Rpl13a snoRNA in a mouse model, while leaving the coding and regulatory regions intact, profoundly influenced mitochondrial metabolism, resulting in enhanced systemic glucose tolerance and protection against oxidative stress (Lee *et al.* 2016). So the delayed inhibition of expression of multiple inflammation - related proteins by the GAIT complex suggests that this system represent a post-transcriptional off - switch that also contributes to inflammation - resolution. The other group that showed altered expression consists of small Cajal body - Specific RNAs (scaRNAs), a sub - group of snoRNAs associated to Cajal body. Cajal bodies, distinct sub - domains of interphase nucleus, represent specific and dynamic assemblies of proteins and RNA factors involved in various aspect of gene expression, such the biogenesis and/or transport of small nuclear ribonucleoproteins (snRNP) and small nucleolar ribonucleoproteins (snoRNPs) (Darzacq *et al.* 2002). It was seen that U85 (over - expressed through our experiment) is able to realize the 2'-O-methylation and psudouridylation of snRNA U5, while U89 and U91 (also over - expressed in our results) could direct post - transcriptional modification of pol II - specific spliceosomal snRNAs, as well as U91 (the only scaRNA over - expressed in our analysis). Following this data, we could speculate that the overall down - regulation of found scaRNAs could impair specific modifications controlled by them, leading the spliceosome to function properly.

5. Conclusions

We realized whole RNA - Seq of two group of RPE cells, treated with oxLDL and untreated, respectively, comparing miRNAs expression changes in four selected time points (1h, 2h, 4h, 6h) over time zero. We found that 84 snoRNAs exhibited expression alterations in treated samples, targeting ribosomal RNA and with host genes involved in several biochemical pathways. One of them, regarding the GAIT mediated translational silencing of ceruloplasmin expression, might be associated for the first time to RP etiopathogenesis. Nevertheless, many other important aspects have to be investigated. Predicted snoRNAs targets resulted from in silico analyses and, even if they are based on statistical significant algorithms and literature data, they will be experimentally validated. Furthermore, a deeper transcriptome sequencing on a larger number of samples could permit us to increase the number of detected snoRNAs, improving the knowledge on regulative functions of these small RNAs and RP.

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