Investigating the modulation of long non-coding RNAs associated with senescence using computational and biological approaches

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Introduction

Aging is a **time-dependent decline of physiological integrity** that leads to cellular and tissue functions impairment [1]. It is the **main risk factor** for high prevalence pathologies such as cancer, cardiovascular and neurodegenerative diseases. The understanding of **how to target aging-associated responses** is essential to slow down aging and to delay age-related disorders. **Senescence** is a **cellular state** characterized by **many molecular alterations**: permanent cell cycle arrest, senescence-associated secretory phenotype (SASP), increased β-galactosidase activity (SA-β-gal) and morphological changes [2]. During aging, senescent cells, friendly called "**zombie**" cells, accumulate causing tissue alterations, and they can be used as **models of aging** [3,4].

Accumulating studies indicate that **long non-coding RNAs (lncRNAs) play important roles in cellular senescence and age-related diseases** at all levels - transcriptional, post-transcriptional, translational, and post-translational - but **their exact biological roles are still elusive** [5,6,7]. **The aim** of the study was to investigate the role of lncRNAs in replicative senescence, by combining **computational and biological approaches**.

Methods and Materials

***In silico* bioinformatics analysis**

- Selection of five publicly available **RNA-Seq datasets related to four cell types** (**Table 1**)
- Differential expression (DE) analysis was performed by a pipeline composed by the following steps: 1. Quality Check - FASTQ files downloaded from SRA were checked with FastQC tool; 2. Mapping & Expression - FASTQ paired-end files were mapped against ENSEMBL genome and transcriptome (release-100) with STAR, combined with RSEM for expression calculation; 3. DESeq2 was used to evaluate statistically significant changes in gene expression, filtered by absolute log2 Fold Change (FC) > 1, adjusted p-value (FDR) < 0.05 and mean read count > 50.

***In vitro* cellular models of replicative senescence (RS)**

- Normal **human** dermal **fibroblasts** (HF) following serial culturing: young (passage 14, n=3) vs. old/senescent (passage 32-33, n=3); **Vascular smooth muscle cells** (VSMC), following prolonged passaging: young (passage 6, n=3) vs. old (passage 16, n=3); Human **umbilical vein endothelial cells** (HUVEC) following prolonged passaging: young (passage 5, n=3) vs. old (passage 21, n=3). Total RNAs and proteins samples were purified from young and old cells following standard methods.
- Old and young **cell characterization** was carried out by multi-biomarker analyses: SA-β-gal staining of fixed cells; protein levels assessment by western blotting of a set of senescence- and SASP-associated proteins (such as CDKN1A(p21), CDKN2A(p16), LMNB1, IL6, CXCL8, MMP3). **Selection of lncRNAs by literature mining**: a panel of lncRNAs was selected from literature based on their relevance and modulation in senescence and in aging.
- **Genes and lncRNAs expression** levels analyses were carried out by **quantitative PCR (qPCR)** based on SYBR™ Green chemistry. Genes and lncRNAs normalization was performed considering the geometric mean of at least two housekeeping genes and data were expressed as Fold Change by using $2^{-\Delta\Delta Ct}$ method [old/young ratio].

Table 1. list of RNA-Seq datasets retrieved from Gene Expression Omnibus (GEO)

RNA-Seq GSE dataset	Samples used in this investigation	#	Citation
GSE155680	HUVEC undergoing replicative senescence (3 old vs 3 young)	6	none
GSE157867	HUVEC undergoing replicative senescence (3 old vs 3 young)	6	Zhang et al. (Aging Cell 2021) PMID:33539668
GSE163251	HUVEC undergoing cell senescence (2 old vs 2 young)	4	Ohori et al. (BMC Genomics 2021) PMID:34856941
GSE171663	VSMC undergoing replicative senescence (3 old vs 3 young)	6	Uryga et al. (Commun Biol 2021) PMID:34021256
GSE63577	Human fibroblasts (HF) cell lines undergoing replicative senescence: BJ (3 old vs 3 young), HFF (3 old vs 3 young), IMR-90 (3 old vs 3 young), Wi-38 (3 old vs 3 young)	24	Marthandan et al. (Biomed Res Int 2015, PMID: 26339636; PLoS One 2016, PMID:27140416)

Results

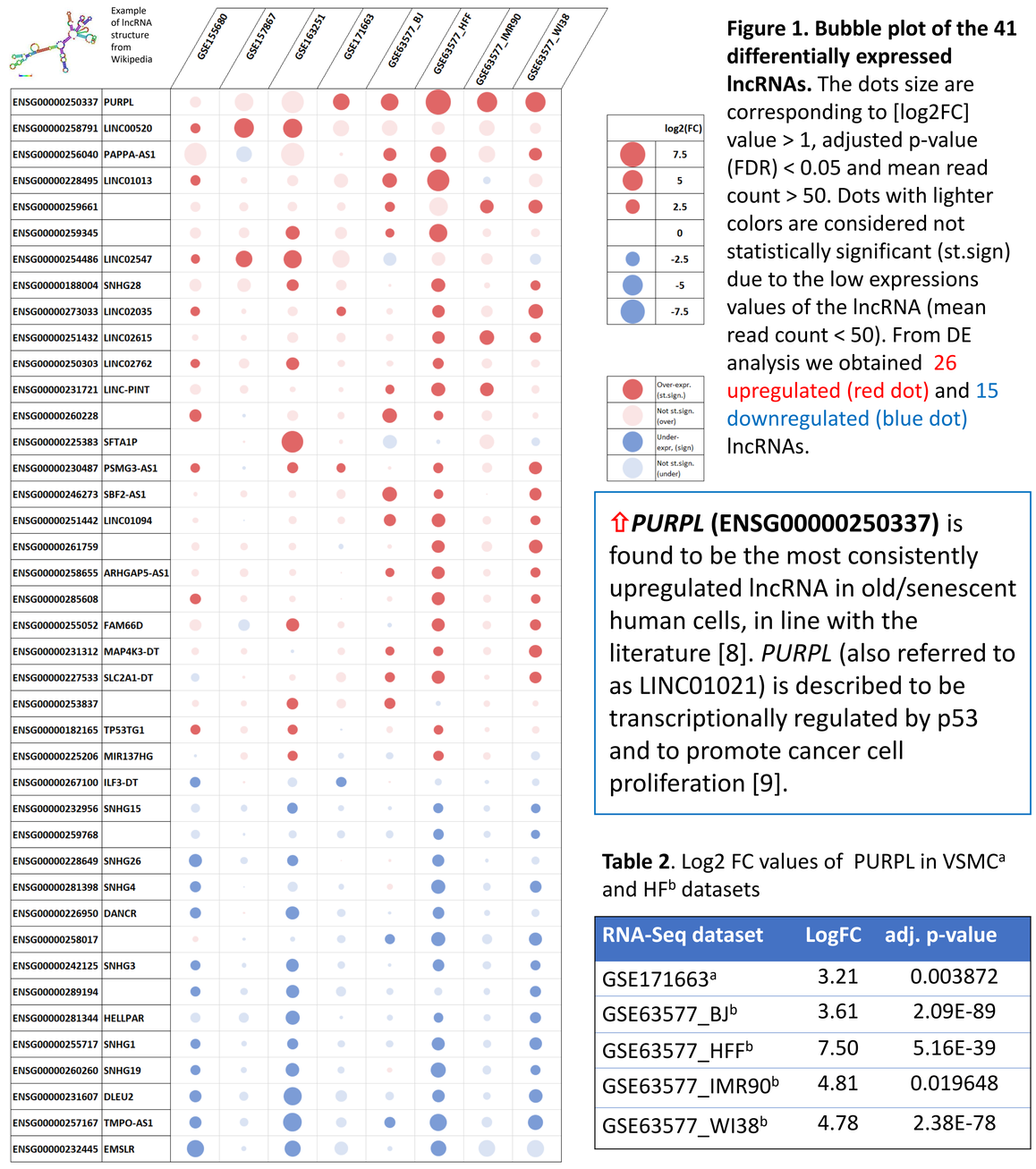

Figure 1. Bubble plot of the 41 differentially expressed lncRNAs. The dots size are corresponding to [log2FC] value > 1, adjusted p-value (FDR) < 0.05 and mean read count > 50. Dots with lighter colors are considered not statistically significant (st.sign) due to the low expressions values of the lncRNA (mean read count < 50). From DE analysis we obtained 26 upregulated (red dot) and 15 downregulated (blue dot) lncRNAs.

⇧***PURPL* (ENSG00000250337)** is found to be the most consistently upregulated lncRNA in old/senescent human cells, in line with the literature [8]. *PURPL* (also referred to as LINC01021) is described to be transcriptionally regulated by p53 and to promote cancer cell proliferation [9].

Table 2. Log2 FC values of PURPL in VSMC[a] and HF[b] datasets

RNA-Seq dataset	LogFC	adj. p-value
GSE171663[a]	3.21	0.003872
GSE63577_BJ[b]	3.61	2.09E-89
GSE63577_HFF[b]	7.50	5.16E-39
GSE63577_IMR90[b]	4.81	0.019648
GSE63577_WI38[b]	4.78	2.38E-78

Table 3. Results of the qPCR analyses of significantly modulated genes and lncRNAs in old cells compared to young cells in three different cell types. FC>2: ⇧upregulation , FC<2: ⇩downregulation

Gene/lncRNAs name	Relevance	HF	VSMC	HUVEC
CDKN1A(p21); *CDKN2A*(p16)	genes-associated senescence	⇧⇧	⇧⇧	⇧⇧
CXCL8, *MMP3*	genes-associated SASP	⇧⇧	⇧⇧	⇧⇧
LMNB1, HMGB1	genes-associated nuclear lamina and DNA	⇩⇩	⇩⇩	⇩⇩
PURPL, *MIR31HG*, *SENEBLOC*	lncRNAs-associated senescence	⇧⇧⇧	⇧⇧⇧	⇧⇧⇩
H19	lncRNA-associated aging and senescence	⇩	⇩	⇩

Conclusions

The DE analysis of publicly available RNA-Seq datasets of human cellular models of RS produced a list of **41 lncRNAs modulated in at least three datasets**. Among them, we found that ***PURPL*** was the most strikingly upregulated, shared by different human cell types undergoing RS (**Table 2**). The *in silico* result was also confirmed by qPCR in our *in vitro* cellular models of RS. This result contributes to validate a previous report [8] and upgrade *PURPL* as a **robust biomarker to characterize senescent cells**. Moreover, focusing on other lncRNAs associated with senescence and aging, we found an upregulation of *MIR31HG* and *SENEBLOC* and a downregulation of *H19* that will deserve further investigations.

Future work

Future work will be aimed at studying the **fine regulatory network involving lncRNAs in senescence** in different cell types and to deeply investigate the role of lncRNAs as sponges for miRNAs by functioning as a competing endogenous RNA's. This interaction will be analyzed by applying miRNA-target prediction tools on our list of senescence associated-lncRNAs and by evaluating which pathways should be influenced by this competition in expression regulation.

References

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