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The LncRNA HOTAIR controls the self renewal, cell senescence, and secretion of antiaging protein α-Klotho in Human Adult Renal Progenitor Cells

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INTRODUCTION

The long no coding RNAs (lncRNA) plays an important role in several biological processes including some renal diseases. Nevertheless, little is know on lncRNA that are expressed in healthy kidney and involved in renal cell homeostasis and development, and even less is know about lncRNA involved in the maintenance of human adult renal stem/progenitor cells (ARPCs) that have been shown to be very important for renal homeostasis and repair processes.

AIM

LncRNAs can be dysregulated in different types of diseases and can also modulate the cellular senescence processes. For the first time we studied their function in ARPCs showing that these cells express high levels of a particular lncRNA, HOTAIR, influencing the cell senescence

METHOD

Whole-genome lncRNA expression was performed by Agilent microarray. lncRNA expression was validated by Real Time PCR. CRISPR/Cas 9 system has been used to knock-down HOTAIR lncRNA. SA-β-Gal experiments were used to evaluate cellular senescence in normal ARPCs and ARPCs knock-out for HOTAIR. By ELISA, it was evaluated the expression of secreted anti-aging protein Klotho. FACS was applied to measure CD133 and protein p15 expression in normal and transfected cells chromatin immunoprecipitation assay (chip) was used to evaluate H3k27me3 in the promoter of p15.

RESULTS

It was found 611 lncRNA specifically expressed in ARPCs compared to RPTECs (Fc>2; FDR<0.05). Among the most significantly modulated lncRNAs, HOTAIR was highly expressed in ARPCs (FC=15; p<0.001). The silenced lines for HOTAIR immediately assumed a senescent phenotype confirmed by the beta-galattosidase assay and decreased proliferation (60% decrease, p<0.001). Moreover, we found that the constitutional, functional, and inverse-senescence marker CD133+ was downregulated in knock-out cells (Fold change=15; p<0.01) and that ARPCs expressed high levels of the α-Klotho anti-aging protein, regulated by HOTAIR exerts its function through the epigenetic silencing of the cell cycle inhibitor p15 inducing the trimethylation of the histone H3k27.

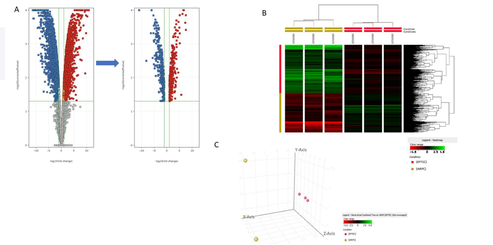

Figure 1. Differences in lncRNA expression between ARPCs and RPTEC. (A) Scatter plot shows genes, miRNA, and lncRNA differentially modulated with FDR < 0.05 and FC > 2. miRNA and genes were then filtered out obtaining the scatter plot representing the lncRNA modulated with a fold change >2. (B) Two-dimensional hierarchical clustering shows that the lncRNA expression profile is different between ARPCs and RPTECs. Four clearly distinct lncRNA clusters were generated. (C) Principal component analysis shows that the 488 lncRNA discriminate the ARPCs from RPTEC.

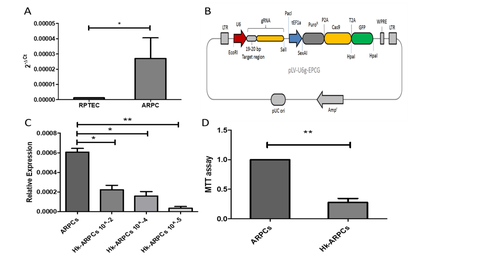

Figure 2. HOTAIR regulates ARPC proliferative ability. (A) Real-time PCR confirmed the high expression of HOTAIR in ARPCs, with a fold change of 22 compared to RPTEC. (B) Vector used for the generation of ARPCs knockout for HOTAIR using the CRISPR/Cas9 technology. The transgene sequence is flanked by long terminal repeat (LTR) sequences (gray box), which facilitate the integration of the transfer plasmid sequences into the host genome. The human U6 promoter is used to drive gRNA expression (red arrow), and the elongation factor 1-alpha (EF1a) (blue arrow) mediates the recruitment of aminoacyl-tRNA to the A-site of the 80S ribosome during protein synthesis. PUROR (gray box) represents the gene for the puromycin resistance, Cas9 (yellow box) represents the recombinant S. pyogenes Cas9 nuclease gene, and GFP (green box) the green fluorescent protein. WPRE (gray box) is the Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element; pUC ori, origin of replication from high copy-number plasmid pUC; Ampr, ampicillin resistance gene (gray boxes). (C) Real-time PCR shows that in ARPCs, HOTAIR expression decreased proportionally using the different lentiviral concentrations, in a range from 10−2 to 10−6 dilutions starting from 106 transduction units (TU)/mL, to transduce cells with the vector for the CRISPR/Cas9 genome editing. (D) MTT assays showed a significant metabolic rate decrease in ARPCs following the HOTAIR knockout.

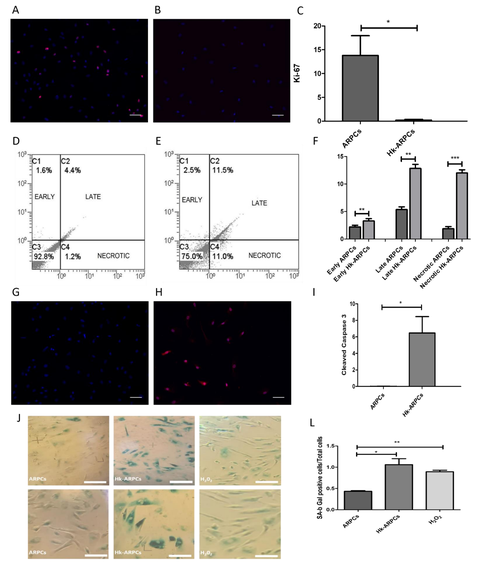

Figure 3. (A, B) Ki-67 (red) immunofluorescence experiments shows a significant decrease in the proliferation marker in Hk-ARPCs (scale bar = 100 μm). DAPI counterstained nuclei (blue). (C) Quantification of immunofluorescence experiments by calculating the pixel ratio of positive cells. HOTAIR affects the expression of CD133 stem cell marker and the senescence in ARPCs. (D) Annexin V/7AAD assay in normal ARPCs. (E) Annexin V/7AAD assay in Hk-ARPCs. Knockout lines showed an increase of ~10% of necrotic ARPCs and an 8% of apoptotic ARPCs, including early and late apoptosis, compared to the basal condition. Results are representative of 5 independent experiments on 5 different cell lines. *P < .05; **P < .01. (F) Statistical analysis of the early, late, and necrosis differences between ARPCs and Hk-ARPCs (**P < .01; ***P < .001). (G, H) Cleaved caspase-3 (red) immunofluorescence experiments shows a significant increase in the apoptosis marker in Hk-ARPCs (scale bar = 100 μm). DAPI counterstained nuclei (blue). (I) Quantification of immunofluorescence experiments by calculating the pixel ratio of positive cells .(J) Following HOTAIR knockout, ARPCs become senescent, as shown by the typical color of SA-β-gal+ cells. The boxes show SA-β-gal assay on non-transduced ARPCs (upper left box), and on Hk-ARPCs (upper middle box). The HOTAIR knockout led to a large number of senescent ARPCs. Cells exposed to H2O2 were used as a positive senescence control (upper right box). Scale bar = 100 μm. Bottom boxes represent magnifications of relative upper boxes. Scale bar = 50 μm. (L) Quantification of SA-β-gal+ cells. The ratio of cells positive to SA-β-gal activity was calculated by examining three non-overlapping fields per condition. *P < .05; **P < .01.

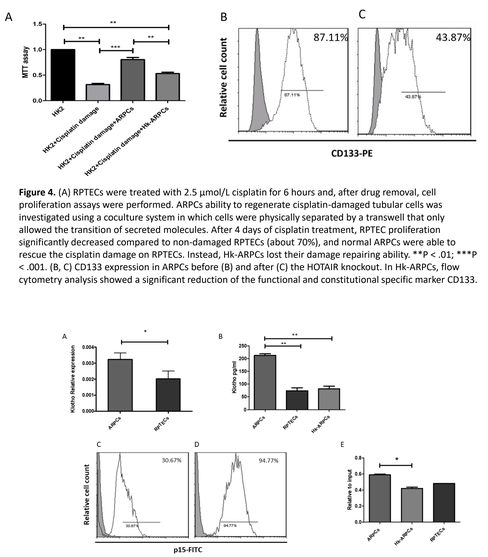

Figure 4. (A) RPTECs were treated with 2.5 μmol/L cisplatin for 6 hours and, after drug removal, cell proliferation assays were performed. ARPCs ability to regenerate cisplatin-damaged tubular cells was investigated using a coculture system in which cells were physically separated by a transwell that only allowed the transition of secreted molecules. After 4 days of cisplatin treatment, RPTEC proliferation significantly decreased compared to non-damaged RPTECs (about 70%), and normal ARPCs were able to rescue the cisplatin damage on RPTECs. Instead, Hk-ARPCs lost their damage repairing ability. **P < .01; ***P < .001. (B, C) CD133 expression in ARPCs before (B) and after (C) the HOTAIR knockout. In Hk-ARPCs, flow cytometry analysis showed a significant reduction of the functional and constitutional specific marker CD133.

Figure 5. HOTAIR influences the expression of Klotho and p15. (A) Klotho expression evaluated by real-time PCR in human amniotic stem cells (hAEC), ARPCs, and RPTEC. Anti-aging protein expression was significantly higher in ARPCs than in other cell lines. (B) α-Klotho expression evaluated by ELISA in supernatants of ARPCs, RPTEC, and Hk-ARPCs. α-Klotho was secreted by ARPCs at significant higher levels compared to that secreted by RPTEC. Klotho levels dropped down in Hk-ARPCs. (C) p15 expression was evaluated by FACS analysis in ARPCs. (D) Following HOTAIR knockout, a significant increase of p15 was found. E) HOTAIR is involved in the epigenetic regulation of the p15 expression. Chromatin from ARPCs, Hk-ARPCs, and RPTEC was immunoprecipitated with anti-H3K27me3 antibody and the H3K27 histone bond in p15 promoter was analyzed by RT-PCR. HOTAIR knockdown weakened the H3K27me3 bond in the promoter of p15. Results are representative of 5 independent experiments on 5 different cell lines. *P < .05; **P < .01.

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CONCLUSIONS

These data demonstrated that HOTAIR regulates the self-renewal capacity of ARPCs and prevents them from becoming senescent in the short term. Moreover, HOTAIR influences ARPCs ability to secrete high levels of α-Klotho, influencing its levels in surrounding tissues modulating, consequently, the kidney aging.

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