Inhibition of miR-21 promotes cellular senescence in NT2-derived astrocytes

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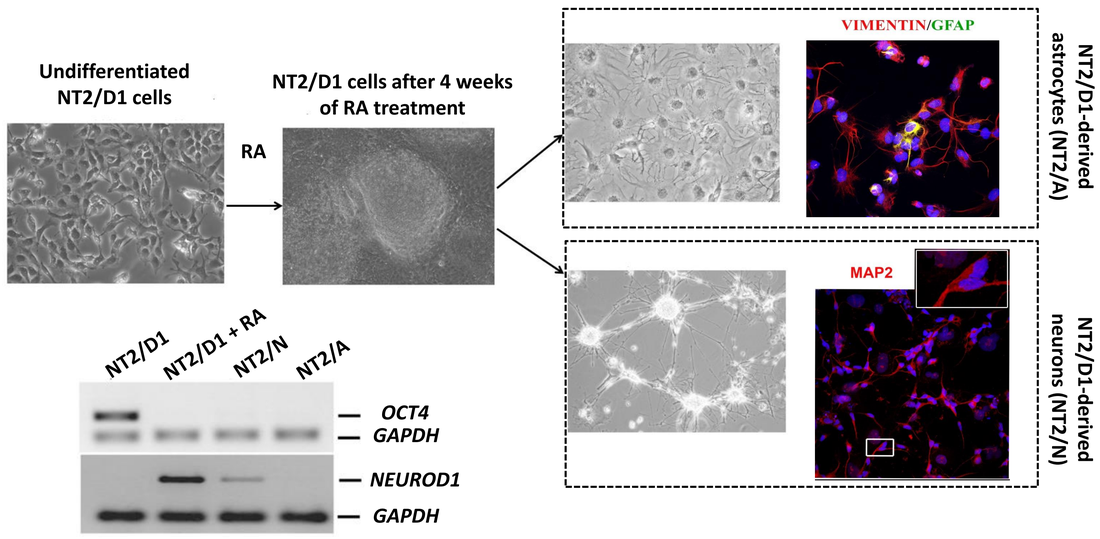
ABSTRACT-Astrocytes are the main homeostatic cells in the central nervous system (CNS). They provide mechanical, metabolic and trophic support to neurons and disruption of their physiological roles can contribute to the CNS dysfunction and pathology. However, the molecular mechanisms underlying their complex physiology are insufficiently explored. Recent studies have shown that miRNAs are involved in the regulation of astrocyte function through different mechanisms. Although miR-21 has been reported as an astrocytic miRNA with an important role in astrogliosis, less is known whether this miRNA also contributes to other phenotypical properties of these cells. We focused our study on miR-21 and its effect on phenotypical characteristics of NT2-derived astrocytes (NT2/A). We down-regulated miR-21 expression in both immature and mature NT2/A by antisense technology. Our results revealed that miR-21 down-regulation induced growth arrest and premature cellular senescence, indicated by senescence hallmarks that include increased expression of cell cycle inhibitors p21 and p53 and augmented senescence-associated β-galactosidase activity, in both immature and mature NT2/A. Additionally, our *in silico* analyses revealed that many of the genes, previously shown to be up-regulated in irradiation-induced senescent astrocytes, were predicted miR-21 targets. Taken together, our results point to miR-21 as a potential regulator of astrocyte senescence. To the best of our knowledge, these are the first data showing the link between miR-21 and cellular senescence of astrocytes. Since senescent astrocytes are associated with different CNS pathologies, development of novel therapeutic strategies based on miRNA manipulation could prevent the senescent state and may improve the physiological outcome.

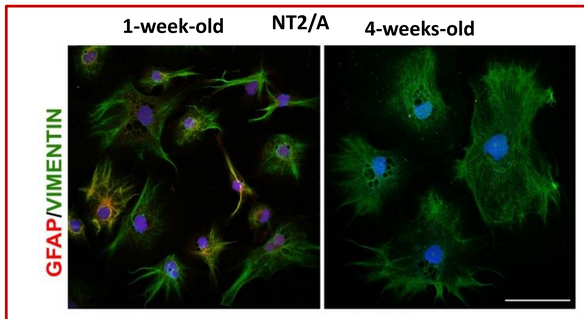
1. Characterization of *In vitro* neural differentiation of human pluripotent NT2/D1 cells

To address the role of miR-21 in astrocytes we used the human pluripotent embryonal teratocarcinoma NT2/D1 cell line as a model system. These cells have the ability to differentiate along the neural lineage during retinoic acid (RA) treatment yielding both neuronal (NT2/N) and glial cell populations (NT2/A) [Pleasure et al.,1992; Sandhu et al.,2002].



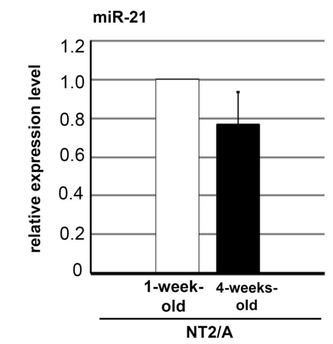
The progression of neural differentiation was documented by changes in cell morphology as well as the expression of specific markers.

2. Characterization of NT2/A in different stages of maturity



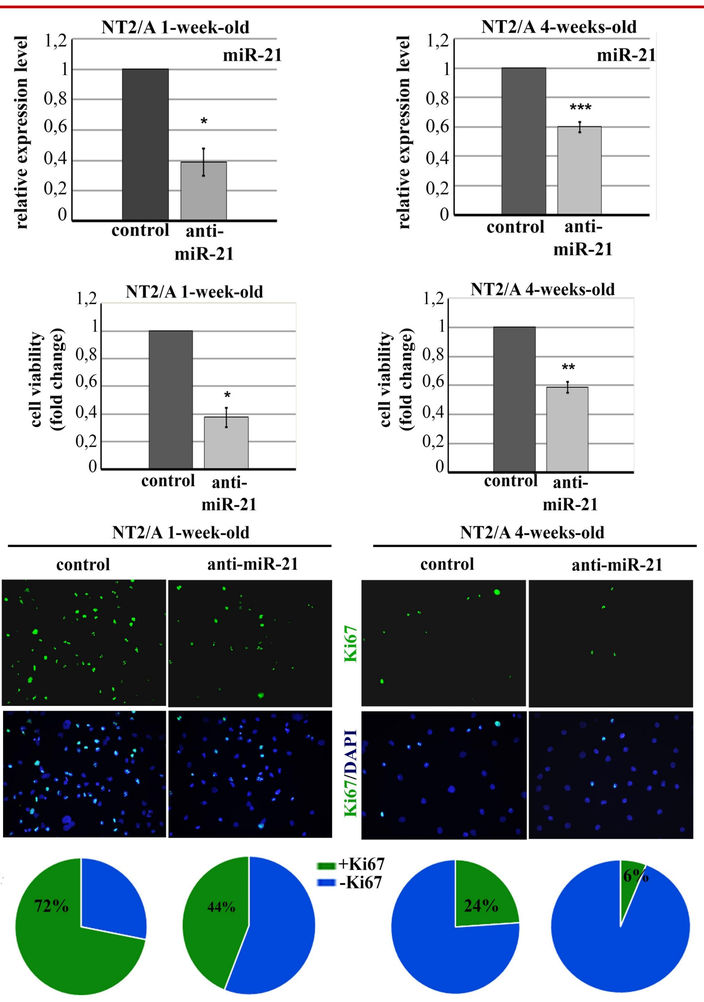
During the *in vitro* maturation of NT2/A changes in cell morphology and the expression of characteristic markers were observed:

One-week-old NT2/A cultures consisted mostly of fibrous astrocytes, positive for both astrocyte-specific markers (GFAP and VIMENTIN), while four-weeks-old NT2/A cultures consisted mostly of VIMENTIN positive protoplasmatic NT2/A.



TaqMan-miR-21 assay results showed a minor, non-significant decrease in the level of miR-21 expression in the four-week-old vs. one-week-old NT2/A, indicating that the level of miR-21 expression remains rather unchanged during the NT2/A maturation.

3. miR-21 down-regulation affects NT2/A proliferation



miR-21 was down-regulated in both immature and mature NT2/A using anti-sense-miR-21 transduction construct (anti-miR-21). Empty vector transduced NT2/A were used as a control.
miR-21 down-regulation was confirmed by qPCR analysis.

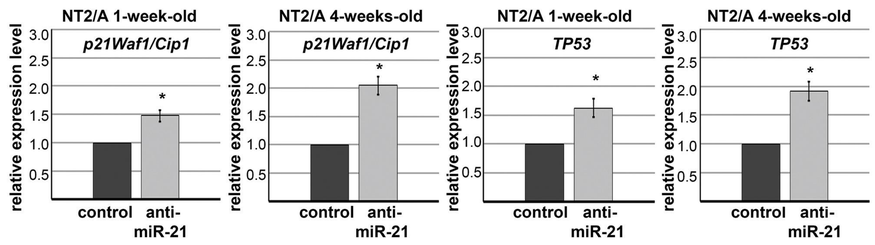
Down-regulation of miR-21 led to a significant decrease in viability of NT2/A at both stages of maturity.
Cell viability was analyzed by MTT assay.

miR-21 down-regulation caused a decrease in the cell proliferation rate of NT2/A at both stages of maturity, as indicated by the decrease in the number of Ki-67 immuno-positive cells.

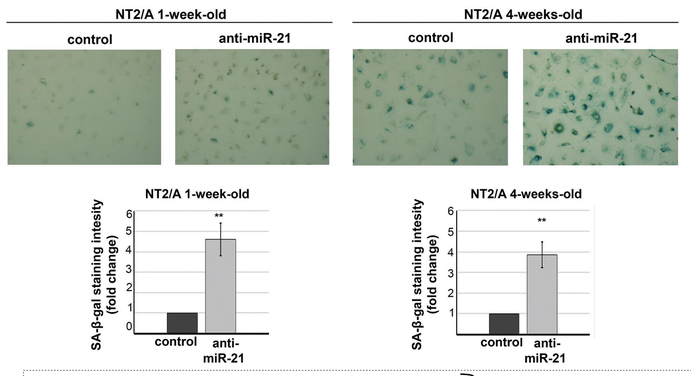
4. miR-21 down-regulation induces NT2/A senescence

miR-21 downregulation induced premature NT2/A senescence, indicated by:

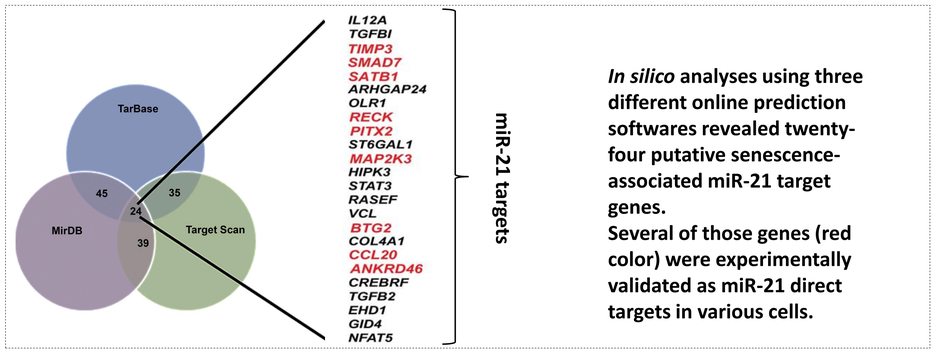
- Increased expression of cell cycle inhibitors p21 and p53 in NT2/A at both stages of maturity.
Expression of these genes was analyzed by qPCR method.



-Increased senescence-associated-ß-galactosidase (SA-ß-gal) activity in NT2/A at both stages of maturity.



Relative increase of SA-β-gal staining intensity in NT2/A transduced with anti-miR-21, was calculated compared with the SA-β-gal staining intensity in control. Quantification of the intensity of SA-ß-gal staining was undertaken using ImageJ software.



In silico analyses using three different online prediction softwares revealed twenty-four putative senescence-associated miR-21 target genes.
Several of those genes (red color) were experimentally validated as miR-21 direct targets in various cells.