Title: ***Investigation of the biological and molecular relevance of NONO protein in multiple myeloma***

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## Introduction and Aims

Multiple myeloma (MM) is a hematological malignancy characterized by the abnormal proliferation of plasma cells (PCs) in the bone marrow (BM) and a highly heterogeneous genetic background[1]. Despite the advancements in therapy, MM still remains an incurable disease[2]. Paraspeckles (PSs) are membraneless subnuclear bodies found in the interchromatin space of mammalian cells involved in many physiological processes, including cellular stress responses, cell differentiation, and cancer progression[3]. In the last years our group strongly contributed to the characterization of the role of PSs in MM[4,5]. PSs assembly relies on the interaction between the lncRNA NEAT1, whose targeting impairs the DNA repair and triggers anti-tumor activity in MM[4], and seven essential paraspeckle proteins (PSPs), including SFPQ and NONO[4,6]. NONO, a multifunctional protein deregulated in many types of cancer, heterodimerizes with SFPQ and binds NEAT1, facilitating PSs assembly[7]. Additionally, NONO is involved in nearly every step of gene regulation, including RNA splicing, DNA unwinding, transcriptional regulation, and the nuclear retention of defective RNA[8]. However, its role in MM remains elusive[3]. We recently demonstrated that CD138+ MM cells exhibit NONO overexpression compared to PCs from healthy donors, emphasizing its association with poor prognosis in MM patients[9]. Therefore, we aim to characterize the biological and molecular functions of NONO in MM, shedding light on its putative role in the pathogenesis of the disease. Finally, our results will potentially contribute to the development of new pharmacological strategies for MM.

## Methods

Gymnotic delivery of a specific LNA-gapmeR (gNONO) was used to silence NONO expression in a panel of 4 human MM cell lines (HMCLs). Dose-effect curves were obtained by Trypan Blue exclusion counts. Cell viability was assessed through CCK-8 assay. $IC_{50}$ values and synergisms evaluation were performed using Compusyn software. Clonogenic potential was evaluated by methylcellulose assay. Cell-cycle phases distribution and apoptosis modulation were investigated by FACS analysis. Cell cycle synchronization was achieved using SynchroSet kit. PS integrity was analysed by confocal microscopy analysis of combined NEAT1 RNA-FISH and NONO immunofluorescence (IF). Western blotting (WB) was used to study PSPs levels.

## Results

To investigate the specific role played by NONO in MM, we exploited a loss of function approach employing the LNA-gapmeR antisense technology by gymnosis. Cells were treated with gapmeRs gNONO and the scrambled gSCR at the same time of seeding.

All the 4 tested HMCLs, albeit at different levels, showed high sensitivity to NONO silencing starting from the 3$^{rd}$ day of gapmeR exposure, as revealed by the $IC_{50}$ value (mean $IC_{50}$ value = 6.5μM).

For all the following silencing experiments we decided to use the sub-cytotoxic 5μM concentration of gNONO which allowed to obtain a significant downregulation of its mRNA expression in all the HMCLs tested (silencing efficiency >80%). Growth curves obtained from the CCK-8 metabolic assay after 7 days of NONO silencing confirmed a significant reduction in the number of viable cells across

all treated samples. Alteration of the proliferative behavior of NONO-depleted cells (NONO-KD) was confirmed by the significant decreased number of colonies (range: 0-11, median = 3) as compared to controls (range: 7-30, median = 25). Morphologic inspections of May-Grunwald stained NONO-KD samples highlighted the appearance of a suffering phenotype, associated with the presence of cytoplasmic vacuoles and membrane blebbing. In line with these observations, cytofluorimetric analyses revealed a time-dependent modulation of cell distribution across different phases of the cell cycle, along with a ≈2-fold increase of the % of cells positive to Annexin V staining upon NONO-KD, confirming apoptosis induction starting from the 4$^{th}$ day from gapmeR delivery. From a molecular point of view, along with the significant downregulation of NONO expression, qRT-PCR results revealed a significant reduction in the expression level of the essential architectural PS scaffold NEAT1 (downregulation of 50-70%, depending on the HMCL), confirming the NEAT1-protective role of NONO also in MM cells. Confocal microscopy experiments confirmed the meaningful reduction of NONO and NEAT1 expression levels, also highlighting a significant decrease of their co-localizing foci, thus demonstrating a strong PSs structure impairment and disruption. Of note, in scramble cells, beside the NONO-NEAT1 co-localizing signals, we highlighted the presence of a diffuse NEAT1-independent NONO staining, thus reinforcing the hypothesis of its PSs-unrelated roles, which deserves further investigations. Finally, WB analysis of NONO-KD cells showed a 2-6-fold increase in the expression levels of two other core-localizing PSPs, namely SFPQ and PSPC1, suggesting a compensatory mechanism between NONO and other PS elements. PSPC1 protein upregulation in NONO-KD cells was confirmed by means of IF in all the tested HMCLs. Additionally, the translational potential of targeting NONO is indicated by its synergistic effects with commonly used MM treatments, such as bortezomib, carfilzomib, melphalan, and olaparib.

**Conclusions**

Our preliminary results highlight the biological significance of NONO in MM cells, warranting further molecular characterization. Overall, our data suggest that NONO targeting could represent a promising strategy for novel anti-MM therapeutic options.


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