Supplementary Data

MiR-93 Attenuates *CALR* Mutant-Driven Oncogenic Activity in Myeloproliferative Neoplasms

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Supplementary Materials and Methods

Advanced Tet-on inducible expression system and luciferase reporter assays

The study employed a Tet-on-advanced vector (C6-4-5 pLAS3w.aOn.Pbsd) and the Tet-on miRNA overexpression cell library (integrated into pAS4w.1.Ppuro) (both purchased from Academia Sinica, Taiwan) to search for *HMGA2* 3'-UTR-targeting miRNAs. Briefly, H293T cells were transfected with the Tet-on plasmid and subjected to blasticidin selection to establish the Tet-on system. Subsequently, the lentiviral vectors within the Tet-on miRNA overexpression cell library system, each encoding one of 594 distinct miRNA overexpressing constructs, were used to transduce the H293T cells harboring the Tet-on system. The entire sequence of the 3'-UTR of *HMGA2* (2999 bp) was PCR amplified using forward Xho1 and reverse Sal1 primers and cloned into the pmirGLO luciferase vector (pmirGLO-*HMGA2*^{WT}-3'-UTR) downstream of the firefly luciferase gene (FL) in a dual-luciferase reporter vector, where the renilla luciferase gene (Rluc) served as a normalization control. Following puromycin selection and clonal amplification, the H293T advanced cells were grown in 96-well plates with or without doxycycline at a concentration of 5 μg/mL. Both the luciferase reporter pmirGLO vector and

the cloned pmirGLO-*HMGA2*^{WT}-3'-UTR vector were transfected into 1 x 10^4 advanced cells using the TransIT-X2® Dynamic Delivery System (Mirus) according to the manufacturer's protocol. Relative luminescence was measured 48 hours post-transfection using a dual-luciferase reporter assay kit (Promega). Luminescence analysis was conducted using a PerkinElmer Infinite 200 plate reader.

To confirm the binding of *miR-93* to the 3'-UTR of *HMGA2*, we engineered *HMGA2*-3'-UTR mutants (*HMGA2*^{MUT}-3'-UTR) by altering the predicted 7bp *miR-93*-targeting sequence "GCACUUU" to "CGUGAAA" via PCR amplification. *HMGA2*^{MUT}-3'-UTR was subsequently cloned into the pmirGLO vector (pmirGLO-*HMGA2*^{MUT}-3'-UTR) and analyzed via luciferase reporter assay as previously described. Primers for construction and PCR amplifying were listed in **Supplemental Table S1**.

MPN cell lines and cell culture

Interleukin-3 (IL-3)-dependent murine Ba/F3 cells with inducible expression of *JAK2*V617F (Ton.JAK2.V617F) or wild-type (WT) JAK2 (Ton.JAK2.WT) were kindly provided by Professor Gregor Hoermann and Professor Matthias Mayerhofer (Medical University of Vienna, Austria) as previously described [1]. *CALR*^{EV}, *CALR*^{WT}, *CALR*^{DEL}, and *CALR*^{INS} cell lines established in the backbone of Ba/F3 cells that also harbored human MPL and both green and red fluorescence were created as previously described [2]. Parental Ba/F3, *CALR*^{EV} and *CALR*^{WT} cells were cultured in media containing 2 ng/ml of IL-3 until 16 hours prior to experiments, whereas both *CALR*^{DEL} and *CALR*^{INS}

cells were maintained in IL-3-free media. All cells were cultured in RPMI-1640 medium (CORNING) supplemented with 10% fetal bovine serum (ThermoFisher) and Antibiotic Antimycotic Solution (100×) (Sigma) at 37 °C in a humidified atmosphere with 5% CO_2.

Viral transduction

To overexpress *miR-93*, we amplified *miR-93* by using forward AgeI linker-primer and reverse EcoRI linker-primer and cloned its sequence into the pLKO-TRC016 plasmid (National RNAi Core Facility, Academia Sinica, Taiwan) to obtain the final product pLKO-*miR93*. The pLKO-TRC016-shScramble plasmid (pLKO-shSC) was used as a negative control. Primers for construction and PCR amplifying were listed in **Supplemental Table S1**. Upon repeated lentiviral infection and antibiotics selection in *CALR*-mutant cells, stable $CALR^{DEL\text{-}miR\text{-}93}$, $CALR^{DEL\text{-}SC}$, $CALR^{INS\text{-}miR\text{-}93}$ and $CALR^{INS\text{-}SC}$ were obtained for subsequent experiments.

Quantitative RT-PCR

To conduct quantitative RT-PCR (qRT-PCR) analysis, we utilized the TOOLSmart RNA Extractor (BIOTOOLS) to isolate total RNA from either murine cell lines or MPN patients' peripheral blood (PB) granulocytes. To quantify the expression level of *miR-93* from murine cell lines, we employed the Mir-X miRNA First-Strand Synthesis kit (Takara Biotechnology) to generate first-strand

complementary DNA (cDNA) from 10 μg of RNA. For measurement of gene expression levels, we applied the Rotor-Gene Q real-time PCR detection system (QIAGEN) and GoTaq® qPCR Master Mix (Promega). To ensure accurate comparisons, we normalized the results to *U6*. To quantify the expression level of *miR-93* from MPN patients, we utilized the TaqMan® Advanced miRNA cDNA Synthesis Kit (ThermoFisher) to synthesize cDNA. The expression levels of *miR-93* were quantified using the TaqMan® Fast Advanced Master Mix (ThermoFisher) and the TaqMan® Advanced miRNA Assay (ThermoFisher), with normalization to the average of miR-320A-3p and miR-423-5p. On quantification of *miR-93* with qRT-PCR, we used mature *miR-93* as the specific 5' primer, and the 3' primer is the mRQ 3' primer supplied from the kit. For the expression of *HMGA2* gene, we utilized the TOOLSQuant II Fast RT Kit (BIOTOOLS) to generate cDNA from 1 μg of RNA and the *HMGA2* gene expression was quantified utilizing the GoTaq® Probe qPCR Master Mix (Promega) and normalized to *GAPDH*. The relative expression levels were determined using the $2^{-\Delta\Delta CT}$ method. Experiments were repeated in triplicates to ensure reliability and reproducibility. Primers for qRT-PCR were listed in **Supplemental Table S2**.

Western blot

Cells were lysed in RIPA buffer (Thermo Scientific), separated by SDS-PAGE, and transferred to membranes. To avoid chemical stripping, membranes were horizontally cut based on pre-stained molecular weight markers prior to antibody incubation. Following hybridization, signals were

visualized via ECL and captured using either traditional X-ray film or a digital chemiluminescence imager. The antibodies utilized were as follows: anti-CALR (208912, Sigma), anti-CALR Mutant (DIA-CAL-250, Dianova), anti-MPL (06-944, Sigma), anti-β-Actin (20536-1-AP, Proteintech), anti-JAK2 (04-001-25UG, Millipore), anti-p-JAK2 (3771, Cell Signaling), anti-STAT3 (12640, Cell Signaling), anti-p-STAT3 (9145, Cell Signaling), and anti-HMGA2 (ab52039, abcam).

Cell viability, proliferation, and apoptosis assays

Cell viability was assessed using the XTT assay according to the manufacturer's instructions (Biological Industries). To evaluate cellular proliferation, cells were cultured for 24 hours, stained with Ki-67 (BioLegend), and analyzed with flow cytometry using the FACS Canto II instrument (BD Biosciences). The percentage of apoptotic cells was determined using flow cytometry with the 7-AAD and APC-Annexin V Apoptosis Detection Kit (BD Biosciences). All experiments were performed in triplicates, and representative data are presented. The green and red fluorescence within the $CALR^{MUT}$ cells we previously constructed allowed us to detect their presence in the PB of transplanted mice and track their proliferation using flow cytometry. To differentiate between live and dead cells, the PB samples of the mice were treated with RBC lysis buffer, re-suspended in PBS, and stained with BD Via-Probe™ Red Nucleic Acid Stain (BD Bioscience).

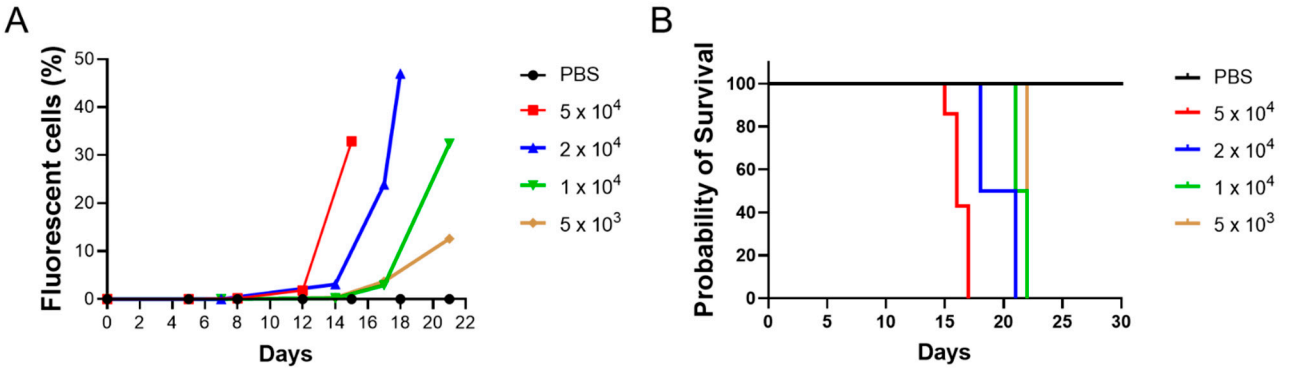
Animals and transplantation of MPN

For replication of diseases in mice, the transplantation encompassed two cell models: $CALR^{DEL}$ and $CALR^{INS}$ MPN. For each model, mice were categorized into four groups based on the cells infused: PBS, $CALR^{MUT}$, $CALR^{MUT\text{-}SC}$, and $CALR^{MUT\text{-}miR\text{-}93}$. Prior to the transplant procedures, busulfan was administered via intraperitoneal injection (I.P.) at the dose of 20mg/kg/day for 5 days (day-6 ~ day-2) to allow for better engraftment of MPN cells. On day 0, a total of $5x10^4$ MPN cells or PBS was infused (via tail vein injection) into each mouse. These mice were then regularly monitored, including daily weight and activity, twice a week blood sampling for complete blood count (CBC) analyses and possible expansion of the MPN clones, subsequent development of apparent illness, and ultimate death. For the detection of MPN clonal expansion, flow cytometry was employed to recognize the emergence of dual-fluorescent MPN cells. In separate cohorts of mice identically transplanted, they were humanely euthanized simultaneously when the first mice succumbed to MPN disease. Following extraction of their spleens and both femurs, comparison was made across different groups with regards to spleen weight, percentage of bone marrow (BM) MPN clonal population, and results of H&E as well as reticulin stains in the BM and spleen.

Tissue staining

The spleens were fixed in 10% neutral buffered formalin, whereas femurs were fixed in 14% formalin, which contained $ZnCl_2$ (80mM $ZnCl_2$, with 0.65% acetic acid). Following fixation for at least 24 h, spleens were de-hydrated and embedded in paraffin. The femur samples underwent decalcification with OSTEOSOFT (Merck), which was followed by paraffin embedment. Tissue sections (4 µm) were then visualized with either H&E staining (Sigma) and Reticulin silver plating kit acc. to Gordon & Sweets (Merck) according to the manufacturer's instructions.

Supplemental Figures



Supplemental Figure 1. Effects on the cell doses for successful engraftment of transplanted MPN cells in ASID mice. Four cell doses were tested in the mice model: $5x10^4$, $2x10^4$, $1x10^4$, and $5x10^3$ MPN cells, with PBS as the control group. Percentages of dual fluorescent cells **(A)** and survival curves **(B)** for the transplanted mice in respective groups were shown. Sample sizes were n = 3 for the PBS control group, and n = 7, 2, 2, 1 for groups transplanted with $5x10^4$, $2x10^4$, $1x10^4$, and $5x10^3$ cells, respectively.

Supplemental Tables

Supplemental Table S1. Primers for construction and PCR amplification

Target	Sequence (5' to 3')
hsa-HMGA2-3'UTR-F	CTCGAGGGGGCGCCAACGTTCGATTT
hsa-HMGA2-3'UTR-R	GTCGACTCTGTTTTGACCAAACTTTA
hsa-HMGA2-3'UTR-R1-F	ATTAAGCATATGTGTTACTTCAAGTAATAC
has-HMGA2-3'UTR-R1-R	TAAAAGTGCAGTTTGAATGAAGAGATGGTG
has-HMGA2-3'UTR-R2-F	TCATTCAAACTGCACTTTTAGCCAGAGATG
has-HMGA2-3'UTR-R2-R	AAATTTTTAATTAAAAAAACATAGAAAAATACATAGTCATG
hsa-HMGA2-3UTR-mut-R1-R	TATTTCACGAGTTTGAATGAAGAGATGGTG
hsa-HMGA2-3UTR-mut-R2-F	TCATTCAAACTCGTGAAATAGCCAGAGATG
hsa-miR-93-F	GCGACCGGTCTGGGGGCTCCAAAGTGCTGTTCGTGCAGGTAGTGTGATTACCCAACCTACTGC
hsa-miR-93-R	CGCGAATTCCCGGGGGCTCGGGAAGTGCTAGCTCAGCAGTAGGTTGGGTAATCACACTACCTG
shRNA-Scramble-F	GCGACCGGTCCGGCCTAAGG
shRNA-Scramble-R	CGCGAATTCAAAAACCTAAGG

Supplemental Table S2. Primers for qRT-PCR.

qPCR primer name	Sequence (5’ to 3’)
hsa-HMGA2 Taqman probe	AGAAGCCACTGGAGAAAAACG
hsa-HMGA2-qPCR-F	AGTCCCTCTAAAGCAGCTCAAAAG
hsa-HMGA2-qPCR-R	GCCATTTCCTAGGTCTGCCTC
hsa-GAPDH-F	TGCCAAATATGATGACATCAAGAA
hsa-GAPDH-R	GGAGTGGGTGTCGCTGTTG
hsa-miR-93-5p	CAAAGUGCUGUUCGUGCAGGUAG
hsa-miR-320A-3p	AAAAGCUGGGUUGAGAGGGCGA
hsa-miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU
ms-miR-93-5P	CAAAGTGCTGTTCGTGCAGGTAG
ms-U6-F	TGGAACGCTTCACGAATTTGCG
ms-U6-R	GGAACGATACAGAGAAGATTAGC

References

1. Chen, C.C.; You, J.Y.; Lung, J.; Huang, C.E.; Chen, Y.Y.; Leu, Y.W.; Ho, H.Y.; Li, C.P.; Lu, C.H.; Lee, K.D.; et al. Aberrant let7a/HMGA2 signaling activity with unique clinical phenotype in JAK2-mutated myeloproliferative neoplasms. *Haematologica* **2017**, *102*, 509-518, doi:10.3324/haematol.2016.154385.
2. Wang, Y.H.; Chen, Y.J.; Lai, Y.H.; Wang, M.C.; Chen, Y.Y.; Wu, Y.Y.; Yang, Y.R.; Tsou, H.Y.; Li, C.P.; Hsu, C.C.; et al. Mutation-Driven S100A8 Overexpression Confers Aberrant Phenotypes in Type 1 CALR-Mutated MPN. *Int J Mol Sci* **2023**, *24*, doi:10.3390/ijms24108747.