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Original Research Article

MiR-92a-1-5p contributes to cellular proliferation and survival in chronic myeloid leukemia and its inhibition enhances imatinib efficacy

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ABSTRACT

Tyrosine kinase inhibitors (TKI), such as imatinib, have revolutionized chronic myeloid leukemia (CML) treatment. Despite this success, TKI intolerance and resistance remain significant clinical challenges. A promising therapeutic approach is to simultaneously target the *BCR::ABL1* oncogene and other oncogenic drivers. The polycistronic *miR-17-92* cluster is known to contribute to CML development and progression, but the specific roles of *miR-92a-1-5p* within this cluster remain unclear. In this study, we assess the roles of this microRNA and evaluate the therapeutic potential of combining microRNA inhibition with imatinib to improve treatment outcome. Our results show that *miR-92a-1-5p* is downregulated by imatinib in myeloid cell lines harboring *BCR::ABL1* and in CML patient samples. Inhibition of *miR-92a-1-5p* reduces proliferation and enhances imatinib-induced cell death, while its overexpression increases proliferation and counteracts the effects of imatinib on cell death. This decrease in proliferation caused by *miR-92a-1-5p* inhibition is rescued after simultaneous inhibition of two newly identified target genes: *BNIP3L (NIX)* and *TP53INP1*. We confirm that *miR-92a-1-5p* regulates proliferation and cell cycle by targeting *TP53INP1* and decreases autophagy by targeting *BNIP3L*. Our data suggest that *miR-92a-1-5p* plays a role in CML progression, and its inhibition enhances imatinib anti-leukemic efficacy, making it a potential therapeutic target.

1. Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm characterized by uncontrolled proliferation of granulocytic progenitor cells, leading to an excess of mature granulocytes in the blood [1]. Essential to disease development is the *BCR::ABL1* oncogene resulting from the translocation t(9;22)(q34;q11). The BCR::ABL1 oncoprotein is a constitutively active tyrosine kinase (TK) driving STAT, MAPK and MYC downstream signaling pathways and promoting growth factor independence and cell proliferation. BCR::ABL1 also phosphorylates STAT5, activates the PI(3)K/AKT pathway and increases BCL-2 expression, resulting in increased resistance of CML progenitors to apoptosis [2,3].

Tyrosine kinase inhibitors (TKI) have revolutionized CML management, with imatinib, the first TKI approved for treatment in 2001 [4]. Traditional TKI target the ATP-binding site of BCR::ABL1, preventing phosphorylation of downstream signaling pathways involved in cellular proliferation and survival [5]. Recently, asciminib, an allosteric inhibitor that binds to the myristoyl pocket and stabilizes BCR::ABL1 in an inactive conformation, was approved for first-line CML therapy [6].

Despite the success of TKI in treating CML, intolerance and resistance remain significant clinical challenges. Treatment discontinuation due to intolerance is reported in 10–20 % of patients, depending on the TKI [7]. Resistance to TKI is classically categorized as primary, occurring in

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15–30 % of patients who fail to achieve an initial response, and secondary, affecting 10–20 % of patients who relapse after an initial response [8]. Up to 60 % of CML patients develop imatinib resistance following *BCR::ABL1* mutations [9]. Other mechanisms include BCR::ABL1 overexpression, increased downstream signaling pathway activation, alterations in drug metabolism and efflux, additional chromosomal abnormalities, epigenetic dysregulation and microenvironmental factors leading to leukemia stem cell persistence and disease progression [10]. The underlying mechanism remains unknown in 10–20 % of cases of TKI resistance.

A promising approach to mitigate TKI complications and resistance involves targeting simultaneously BCR::ABL1 and other oncogenes implicated in disease development. In this context, we explore the potential of targeting oncogenic microRNAs (miRNAs), also known as oncomiRs. MiRNAs are small, single-stranded, non-coding RNAs involved in the post-transcriptional regulation of target mRNA through selectively binding to their 3′-untranslated region (3′UTR) and blocking protein expression. One miRNA can bind to as many as 200 target genes, and collectively, miRNAs are reported to control about 60 % of human genes. Considering their numerous roles in regulating crucial cellular processes and ability to simultaneously target multiple signaling pathways, miRNAs are promising therapeutic targets [11,12].

The *miR-17-92* oncogenic cluster (*miR-17*, *miR-18a*, *miR-19a*, *miR-20a*, *miR-19b-1* and *miR-92a-1-5p*) has been reported to participate in the pathogenesis of multiple diseases including leukemias, solid cancers, cardiovascular and neurodegenerative diseases [13]. The cluster and its members regulate cell cycle and apoptosis pathways by targeting key factors like E2F1-3, CDKN1A (p21), and BCL2L11 (BIM) [14]. In the

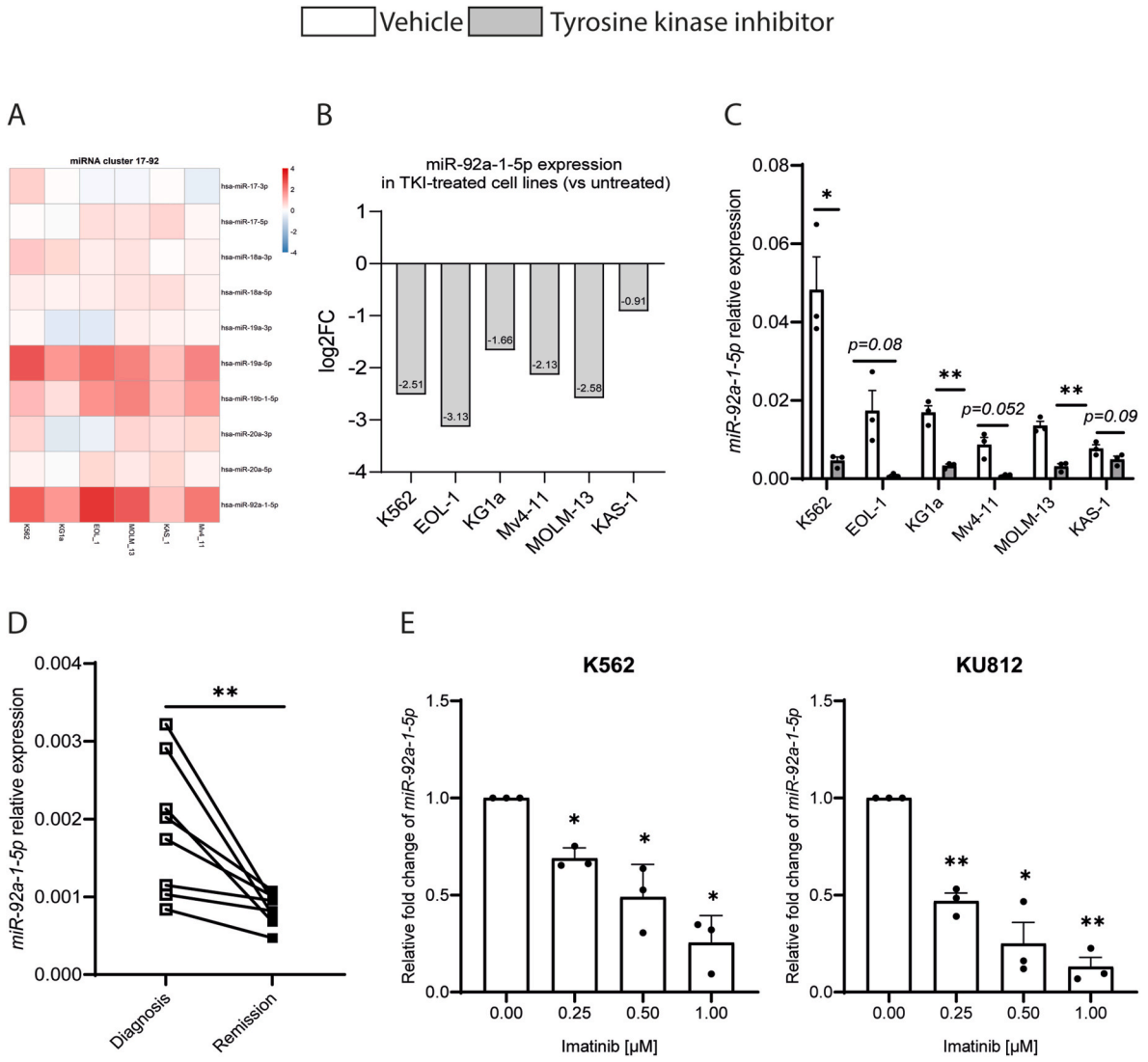

Fig. 1. Expression of *miR-92a-1-5p* decreases under tyrosine kinase inhibitor treatment in myeloid cell lines and in CML patient samples. Small RNA sequencing was performed on six acute myeloid leukemia cell lines (K562, EOL-1, KG1a, Mv4-11, MOLM-13 and KAS-1), untreated or treated with the effective tyrosine kinase inhibitor as described in Supplementary Table 3. Heatmap of log 2-fold change values in the expression of miRNAs from the *miR-17-92* cluster, comparing untreated and TKI-treated acute myeloid leukemia cell lines. Red indicates upregulated miRNAs in untreated cells, blue indicates upregulated miRNAs in TKI-treated cells and white indicates no significant differential expression **(A)**. Bar graph represents *miR-92a-1-5p* expression in all six cell lines, treated vs untreated (Log2FC) **(B)**. Expression of *miR-92a-1-5p* was measured by RT-qPCR and normalized to the expression of *RNU44* in six myeloid cell lines treated or not with the effective tyrosine kinase inhibitor, as described above (N = 3; unpaired Student *t*-test with or without Welch correction: *p < 0.05; **p < 0.01) **(C)**. RT-qPCR analysis of *miR-92a-1-5p* in 8 bone marrow samples from *BCR::ABL1* + CML patients at diagnosis (before treatment) and in complete molecular remission (under treatment) and normalized to the expression of *RNU44* (paired Student *t*-test: **p < 0.01) **(D)**. K562 (left) and KU812 (right) cells were treated with increasing concentrations of imatinib. Expression of *miR-92a-1-5p* was measured by RT-qPCR and normalized to the expression of *U6* and expressed compared to the data from untreated cells (N = 3; One-sample *t*-test: *p < 0.05) **(E)**.

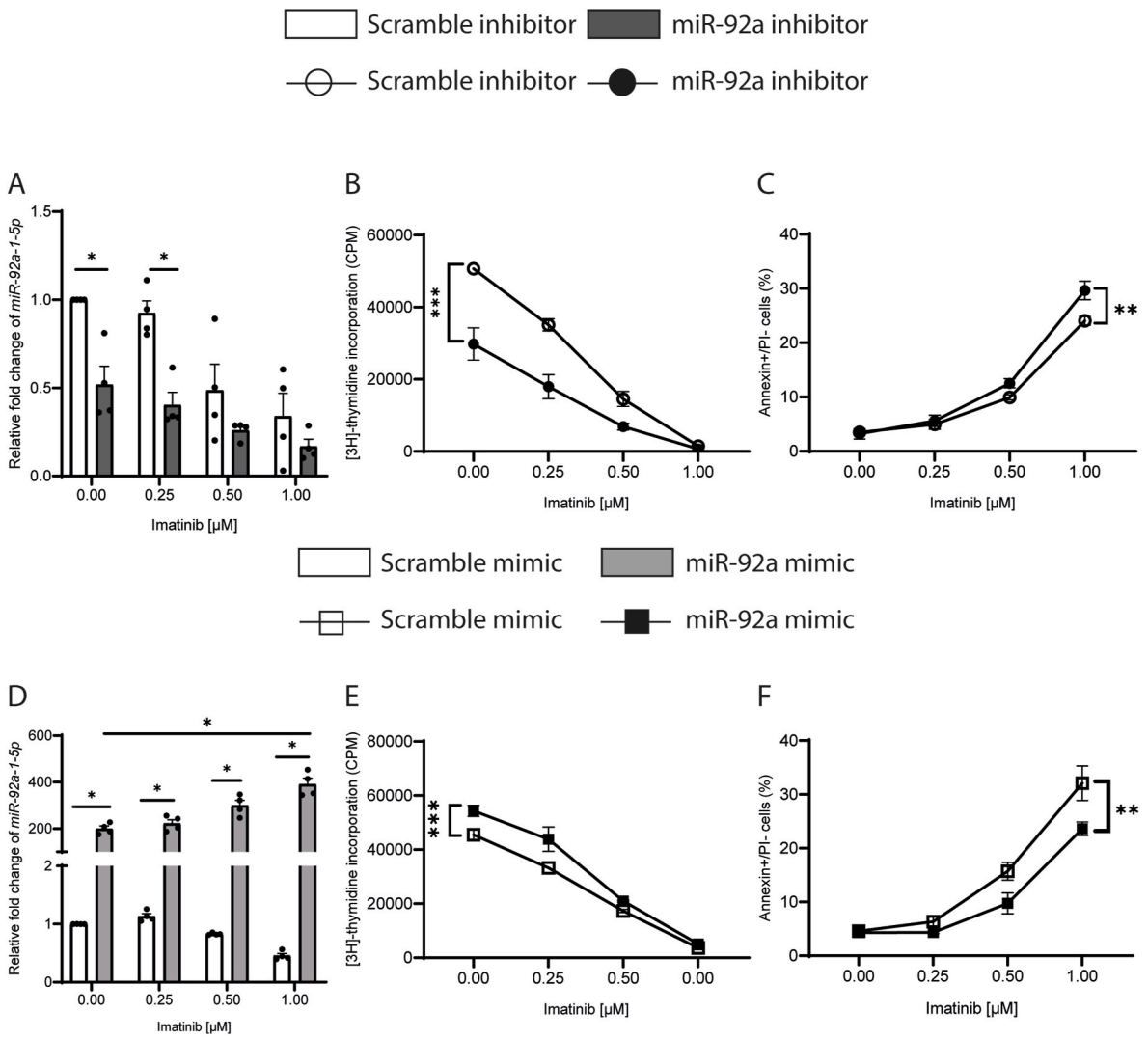

Fig. 2. Inhibition and overexpression of *miR-92a-1-5p* affects cellular proliferation with or without imatinib treatment as well as apoptosis under treatment. K562 cells were transfected with either miR-92a inhibitor **(A, B & C)** or miR-92a mimic **(D, E & F)** or their respective controls. After 24h, *miR-92a-1-5p* expression was measured by RT-qPCR, normalized to the expression of *U6* and expressed compared to the data from untreated control cells (Mann-Whitney test N = 4; *p < 0.05) **(A & D)**. 48h post transfection, cellular proliferation was analyzed by measuring [3H]-thymidine incorporation (count per minutes, CPM) **(B & E)** and cellular apoptosis was measured by flow cytometry using Annexin V and propidium iodide staining. The results represent the percentage of apoptotic cells **(C & F)** For panels B, C, E and F, statistical comparison was performed with the Two-way ANOVA test (N ≥ 3; *p < 0.05; **p < 0.01).

K562 cell line, a model for CML, overexpression of the cluster as a whole increased cell proliferation while its genomic depletion inhibited the *BCR::ABL1* induced leukemogenesis in mice transplanted with *BCR::ABL1* transduced hematopoietic stem cells [15,16]. Given their oncogenic potential, we hypothesized that the *miR-17-92* cluster or its individual members could serve as promising targets for novel therapeutic strategies. Inhibiting miRNAs of the cluster may reduce leukemic cell proliferation, enhance TKI efficacy, and potentially overcome resistance.

In this study, we assessed the previously undescribed roles of *miR-92a-1-5p,* a member of the *miR-17-92* cluster, in CML development and progression and examined the therapeutic potential of *miR-92a-1-5p* inhibition in combination with imatinib. We identified and validated two novel target genes of the miRNA, involved in cell cycle and autophagy respectively. Our data strongly support that combining imatinib with targeted miRNA therapy enhances its efficacy.

2. Methods

2.1. Cell culture

Human AML cell lines described in Supplementary Table 3 were purchased from DSMZ and cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640, Lonza) supplemented with 10 % fetal bovine serum (FBS, Cytiva), 50 U/mL penicillin and 50 mg/mL streptomycin (Gibco). KU812 cells were purchased from DSMZ and cultured as above. HEK-293T cells (ATCC) were cultured in Dulbecco Modified Eagle Medium (DMEM, Gibco) supplemented as described above.

2.2. Cell transient transfection

Cells were nucleoporated as previously described [17] with synthetic miRNA and siRNA described in Supplementary Table 1.

2.3. Proliferation and apoptosis assay

Cell proliferation was studied by [3H]-thymidine (0.5 mCi, Revvity)

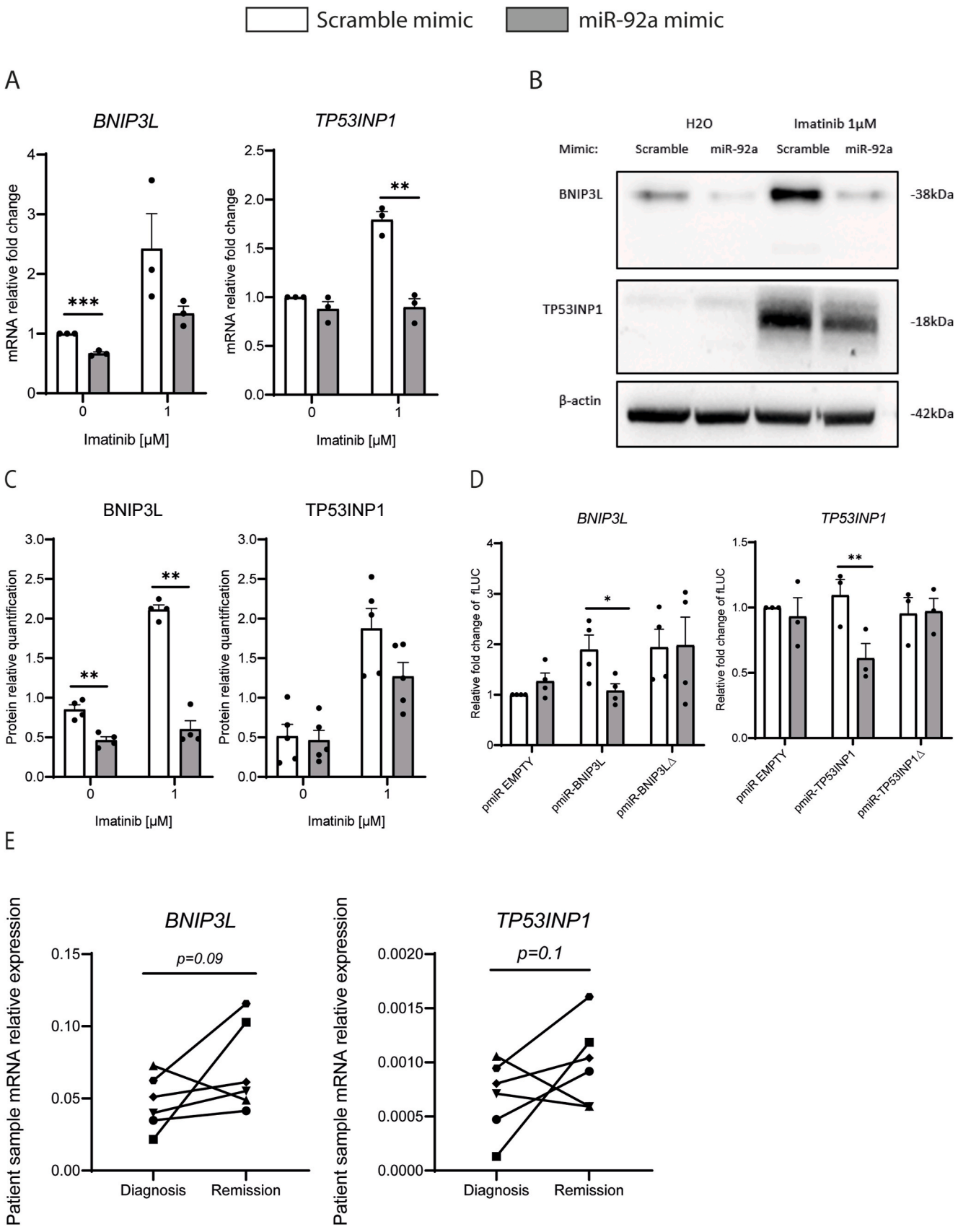

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Fig. 3. Overexpression of *miR-92a-1-5p* directly modifies the expression of *BNIP3L* and *TP53INP1* at mRNA and protein levels. K562 cells were transfected with scramble/miR-92a mimic and treated with imatinib. 24h post transfection, expression of *BNIP3L* (left) and *TP53INP1* (right) was measured by RT-qPCR, normalized to the expression of *RPLP0* and expressed compared to the data from untreated control cells (unpaired Student *t*-test N = 3; *p < 0.05, **p < 0.01) **(A)**. At the same time-point, protein expression of BNIP3L and TP53INP1 was measured by western-blot (image representative of 4 replicates) **(B)**. Quantification of BNIP3L and TP53INP1 protein levels was normalized to β-actin and expressed compared to the data from untreated control cells (N = 4; unpaired Student *t*-test: **p < 0.01) **(C)**. HEK-293T cells were transfected with normal and mutated (Δ) luciferase pmiR-BNIP3L and pmiR-TP53INP1 constructs, miR-92a mimic as well as the appropriate controls. Luciferase activity was measured at 24 h, normalized using beta-galactosidase and expressed compared to the data from control cells (N ≥ 4; unpaired Student *t*-test with or without Welch correction: **p < 0.01) **(D)**. Expression of *BNIP3L* (left) and *TP53INP1* (right) was measured by RT-qPCR, normalized to the expression of *RPLP0* in patient samples at diagnosis and in remission under imatinib treatment. No significance was reached with the paired Student *t*-test (N = 6) **(E)**.

incorporation as previously described [18]. Radioactive activity was measured using a MicroBeta2 Microplate Counter (PerkinElmer). Cell apoptosis was quantified by flow cytometry (BD FACSVerse Cell Analyser, BD Bioscience) using the FITC Annexin V Apoptosis detection Kit II (BD Bioscience).

2.4. Luciferase assays to determine miR-92a-1-5p target genes

HEK-293T cells were co-transfected through the calcium phosphate method [19], with 150 ng pMirTarget (OriGene) constructs containing the wild-type or mutated (Δ) 3′UTR segment of target genes (pMiRTarget_BNIP3L, pMiRTarget_TP53INP1, pMiRTarget_BNIP3LΔ, pMiRTarget_TP53INP1Δ), pEF1-β-galactosidase (0.3 μg, Invitrogen) and 100 pmol of hsa-miR-92a-1-5p mimic and firefly luciferase activity was monitored [18].

2.5. Cell cycle assay

Cells were fixed 30 min at 4 °C in PBS-EtOH 70 %, treated with RNAse A (100 μg/mL) (Thermo Scientific) 5 min at room temperature and stained with propidium iodide (50 μg/mL) (Molecular probes, Life technologies). Cell cycle distribution was measured by flow cytometry and analyzed with FlowJo.

2.6. Immunofluorescence

Treated (10 μM of chloroquine) or untreated cells were immobilized on coverslips pre-coated with Poly-L-lysine solution (Sigma-Aldrich) 48 h post-transfection. Followed by 10 min fixation (4 % paraformaldehyde, 2 % sucrose, PBS), 30 min permeabilization (Tween-20 0.5 %, PBS-BSA 5 %), 1h blocking (PBS-BSA 1 %) and overnight incubation at 4 °C with the indicated primary antibodies diluted in PBS-BSA 1 % (Supplementary Table 2) and Hoechst 33258, pentahydrate (Invitrogen) for nucleus staining, coverslips were mounted using Dako Fluorescence mounting medium (Agilent Technologies). Images were acquired using Zeiss Cell Observer Spinning Disk (COSD) confocal microscope using a plan-Apochromat 100× NA 1.4 oil immersion objective and the same settings for illumination. Images were analyzed using Zen software.

2.7. Statistical analyses

Data are expressed as means ± SEM when the number of independent experiments was N ≥ 3 or means ± SD if N ≤ 2. For all experiments, statistical tests were performed only when N ≥ 3. When comparing 2 unpaired populations: unpaired Student *t*-test was performed with or without Welch correction, as well as the Mann-Whitney test for populations without a Gaussian distribution. For the patient samples, paired Student *t*-tests were performed. One-sample *t*-test was performed when comparing multiple populations to one control population. For multiple comparisons, One-way ANOVA tests were performed with or without Brown-Forsythe and Welch correction, as well as Two-Way ANOVA tests. *p < 0.05, **p < 0.01, ****p < 0.0001, non-significance is not shown on the graph.

3. Results

3.1. MiR-92a-1-5p expression decreases under TKI treatment in myeloid cell lines and in CML patients

Small RNA sequencing was performed on six acute myeloid leukemia cell lines presenting TK alterations and treated with their appropriate TKI at clinically relevant concentrations (Supplementary Table 3). *MiR-92a-1-5p* was preselected based on its fold change across all six cell lines (Fig. 1A and B). Differential expression of *miR-92a-1-5p* in these AML cell lines under TKI treatment was validated by RT-qPCR (Fig. 1C). *MiR-92a-1-5p* expression was measured in bone marrow samples from CML patients at diagnosis in chronic phase and in remission under imatinib treatment. A significant decrease in *miR-92a-1-5p* expression was found under treatment (Fig. 1D). A gradual decrease in *miR-92a-1-5p* expression was confirmed in two *BCR::ABL1* positive cell lines, K562 and KU812, when treated with increasing concentrations of imatinib (Fig. 1E). *MiR-92a-1-5p* expression was also decreased in K562 cells following treatment with dasatinib or nilotinib, two-second-generation TKIs (Supplementary Fig. 2).

3.2. MiR-92a-1-5p inhibition decreases cellular proliferation and increases apoptosis in combination with imatinib

To investigate the impact of *miR-92a-1-5p* on cellular processes, we down-regulated or up-regulated *miR-92a-1-5p* expression through transient transfection of K562 cells with synthetic miRNA inhibitors or mimics. Inhibition of *miR-92a-1-5p* led to a reduction in its expression, similar to the effect observed with 0.5 μM imatinib treatment (Fig. 2A). Combining *miR-92a-1-5p* inhibition with imatinib treatment further decreased miRNA expression levels (Fig. 2A). Functionally, *miR-92a-1-5p* inhibition alone decreases cellular proliferation, with enhanced effects when adding increasing concentrations of imatinib treatment (Fig. 2B). Annexin-V and Propidium iodide staining revealed that inhibition of *miR-92a-1-5p* combined with 0.5 and 1 μM of imatinib treatment increases the proportion of apoptotic cells (Fig. 2C). Given the *miR-17-92* cluster's role in targeting cell cycle and apoptosis regulators, *miR-92a-1-5p* downregulation may enhance imatinib-induced cell cycle arrest and apoptosis.

In the absence of imatinib, transfection of the miR-92a mimic resulted in a 200-fold increase in *miR-92a-1-5p* expression. *MiR-92a-1-5p* expression was further increased in transfected cells treated with imatinib in a dose-dependent manner, with a significant upregulation observed at 1 μM compared to untreated transfected cells (Fig. 2D). Cells overexpressing *miR-92a-1-5p* exhibited increased proliferation, even when treated with imatinib at concentration up to 0.5 μM (Fig. 2E). Additionally, *miR-92a-1-5p* overexpression combined with imatinib treatment reduced the proportion of Annexin-V+/PI- apoptotic cells (Fig. 2F), supporting its role in promoting cell proliferation and suppressing apoptosis.

3.3. BNIP3L and TP53INP1, two newly identified target genes of miR-92a-1-5p

To gain a deeper comprehension of *miR-92-1-5p* functions in CML,

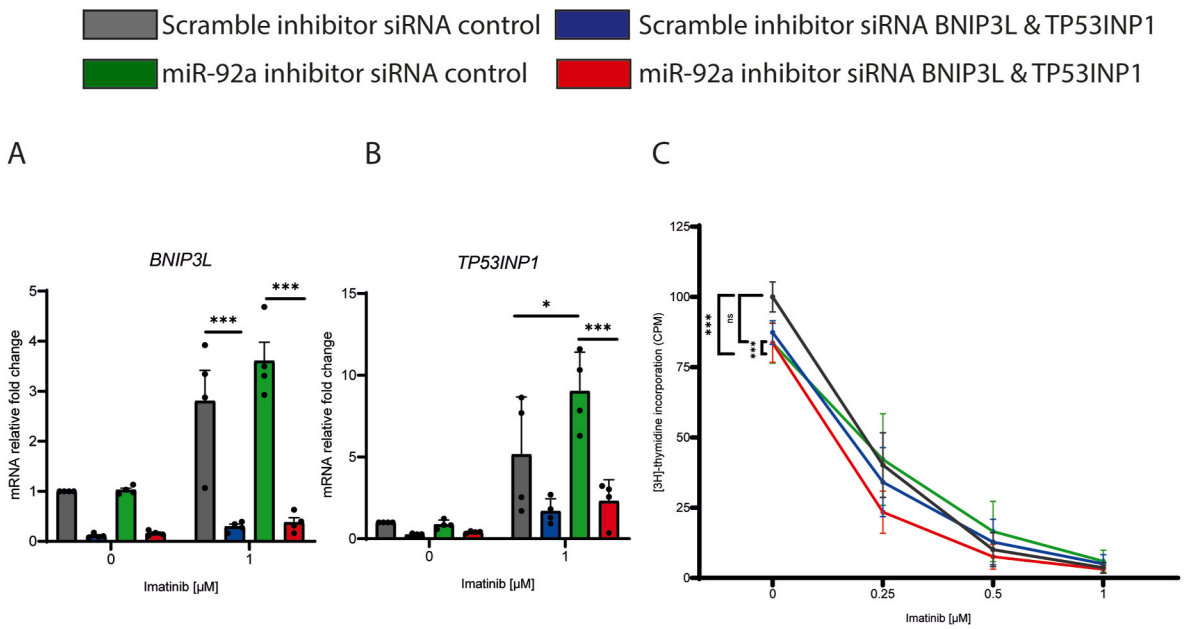

Fig. 4. Decrease in proliferation induced by *miR-92a-1-5p* inhibition is rescued when *BNIP3L* and *TP53INP1* expression is knocked down. K562 cells were transfected with miR-92a inhibitor, BNIP3L & TP53INP1 siRNAs and the appropriate controls. *BNIP3L* and *TP53INP1* expression was measured by RT-qPCR 24h after transfection, normalized to the expression of *RPLP0* and expressed compared to the data from untreated control cells **(A & B)**. Cellular proliferation was analyzed 24h post transfection by measuring [3H] thymidine incorporation (CPM) and expressed compared to the data from untreated control cells. Data are shown in percentages **(C)**. For panels A & B, statistical comparison was performed with the One-way ANOVA test and for panel C with the Two-way ANOVA test (N ≥ 3: *p < 0.05; **p < 0.01; ***p < 0.001).

we sought to identify novel target genes potentially involved in disease development. RNA sequencing was performed on imatinib-treated K562 cells transfected with either scramble or miR-92a mimic. Potential target genes were selected based on their Log2 fold change, statistically significant adjusted p-value, and pathophysiological relevance, with a focus on cellular proliferation, apoptosis and autophagy. The preselected candidates were cross-referenced with the TargetScan database to retain only those predicted to be biological targets of *miR-92a-1-5p* [20]. Two previously unreported genes were selected: *BNIP3L* an autophagy and apoptosis regulator and *TP53INP1* a tumor suppressor regulating cell cycle arrest [21,22]. Imatinib treatment alone, which lowered *miR-92a-1-5p* expression as previously mentioned, increased *BNIP3L* and *TP53INP1* mRNA expression levels (Fig. 3A). *MiR-92a-1-5p* overexpression decreased *BNIP3L* mRNA levels independently of imatinib treatment (Fig. 3A - left), but only decreased *TP53INP1* mRNA when combined with imatinib (Fig. 3A - right). This was due to the low baseline expression of *TP53INP1* under normal conditions, which increases in response to cellular stress [22], as confirmed by western blot (Fig. 3B) with protein quantification (Fig. 3C).

To confirm whether *miR-92a-1-5p* directly regulates *BNIP3L* and *TP53INP1* expression, luciferase assays were performed with expression driven by the regulation of the downstream cloned 3′UTR region. The 3′UTR sequences of *BNIP3L* and *TP53INP1* containing the predicted *miR-92a-1-5p* binding site were inserted into a pMIR-luciferase vector (pmiR-

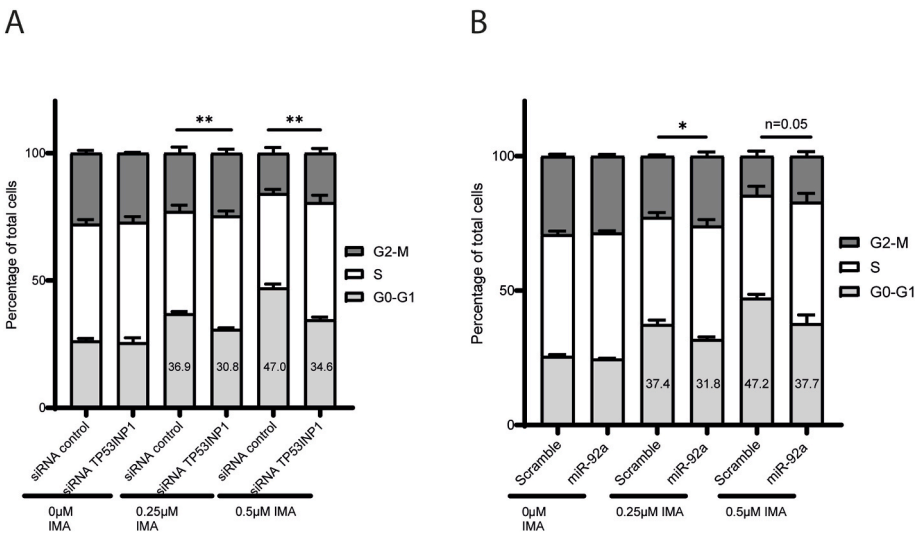

Fig. 5. *MiR-92a-1-5p* overexpression reproduces the action of *TP53INP1* inhibition on cell cycle distribution. K562 cells were transfected with siRNA control/TP53INP1 **(A)** or scramble/mir-92a mimic **(B)** and treated with increasing concentrations of imatinib. Cell cycle distribution was measured 24h post transfection through PI staining and flow cytometry. (N = 3; unpaired Student *t*-test: *p < 0.05; **p < 0.01).

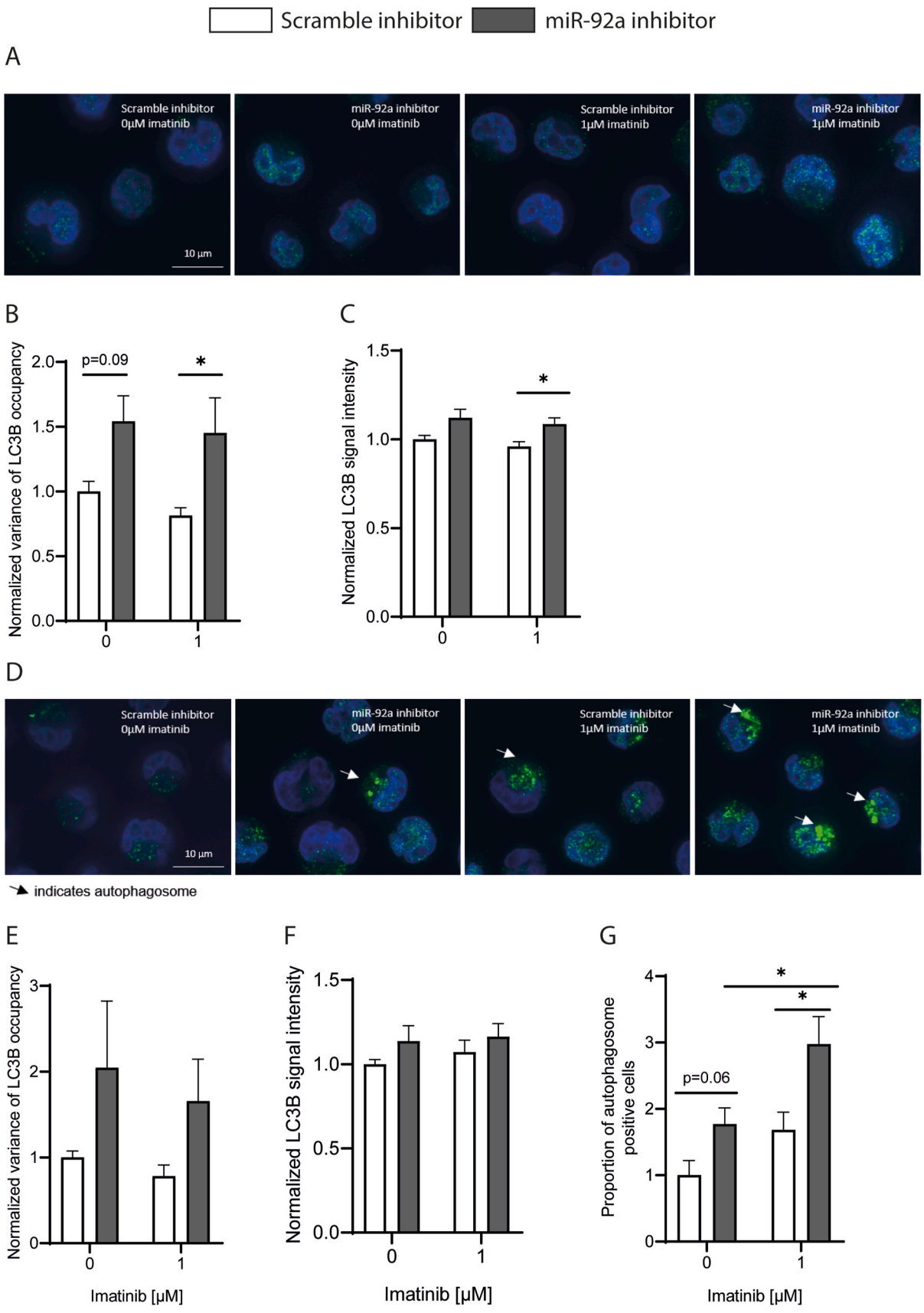

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Fig. 6. Inhibition of *miR-92a-1-5p* increases the number of autophagosome positive cells. K562 cells were transfected with scramble/miR-92a inhibitor. Forty hours later, cells were treated with imatinib for 8h. Immunofluorescence assay was performed on LC3B (in green). The nucleus was colorized by Hoechst **(A)**. Images were analyzed with the Fiji software. Variance of LC3B occupancy and LC3B signal intensity were quantified (N = 3; 10 images analyzed per experiment: Mann-Whitney test: *p < 0.05; **p < 0.01) **(B & C)**. K562 cells were transfected with scramble/miR-92a inhibitor and treated with 10 μM of chloroquine. Forty hours later, cells were treated with imatinib for 8h. Immunofluorescence assay was performed on LC3B (in green). The nucleus was colorized by Hoechst **(D)**. Images were analyzed with the Fiji software. Variance of LC3B occupancy and LC3B signal intensity were quantified (N = 3; 10 images analyzed per experiment: no significance was detected with the Mann-Whitney test) **(E & F)**. Images were analyzed with the Fiji software. Autophagosome positive cells were quantified and normalized to the total number of cells (N = 3; 10 images analyzed per experiment: One-way ANOVA test with Brown-Forsythe and Welch correction: *p < 0.05) **(G)**.

BNIP3L and pmiR-TP53INP1). The predicted binding site was deleted through site-directed mutagenesis to demonstrate direct binding of *miR-92a-1-5p* (pmiR-BNIP3LΔ and pmiR-TP53INP1Δ). Luciferase activity was measured after co-transfecting luciferase vectors (wild-type or deleted) and scramble/miR-92a mimic into HEK-293T cells. *MiR-92a-1-5p* overexpression significantly decreased pmiR-BNIP3L and pmiR-TP53INP1 luciferase activity, which was restored after deletion of the predicted binding site (Fig. 3D). These results demonstrated that *miR-92a-1-5p* directly binds to the 3′UTR region of the two new target genes *BNIP3L* and *TP53INP1*. *BNIP3L* and *TP53INP1* mRNA expression levels increased in patients during remission (under imatinib treatment) compared to diagnosis (Fig. 3E).

3.4. MiR-92a-1-5p influences cellular proliferation through BNIP3L and TP53INP1 downregulation

MiR-92a-1-5p inhibition alone or in combination with imatinib significantly reduced cellular proliferation (Fig. 2B). To confirm the role of *BNIP3L* and *TP53INP1* in this function, we combined miR-92a inhibitor with siRNAs targeting these genes. Expression levels of both targets increased with imatinib treatment alone or combined with miR-92a inhibitor and decreased after siRNA transfection (Fig. 4A and B). The inhibition of *BNIP3L* and *TP53INP1* by siRNAs rescued the decrease in proliferation induced by *miR-92a-1-5p* inhibition back to control levels (Fig. 4C). This was only observed under imatinib treatment, probably because *BNIP3L* and *TP53INP1* basal expression levels were too low and increased under cellular stress, as previously reported [21, 23]. The individual knockdown of *TP53INP1* restored cellular proliferation levels, while the knockdown of *BNIP3L* did not (Supplementary Fig. 5A and B).

3.5. MiR-92a-1-5p decreases cell cycle arrest through TP53INP1 regulation

Overexpression of *TP53INP1* promoted cell cycle arrest in G1 phase in HEK293T cells but its role in CML remains unclear [22]. To investigate this, cells were transfected with siRNAs targeting *TP53INP1* or miR-92a mimic and treated with imatinib, cell cycle distribution was measured through PI staining. PI fluorescence intensity detected by flow cytometry analysis correlated with DNA content, allowing differentiation of cell cycle phases: G0/G1 phase (low PI intensity), S phase (intermediate intensity) and G2/M phase (high intensity). Imatinib treatment alone increased the number of cells in G0-G1 phase, while *TP53INP1* downregulation by siRNAs in treated cells decreased the number of cells in cell cycle arrest (Fig. 5A). Similar results were obtained by *miR-92a-1-5p* overexpression (Fig. 5B).

3.6. MiR-92a-1-5p inhibition induces autophagy

BNIP3L promotes both apoptosis and autophagy by forming homodimers at the mitochondrial outer membrane following cellular stress [21]. The potential role of *miR-92a-1-5p* in autophagy remains unclear. To explore this, immunofluorescence assays were conducted on K562 cells transfected with miR-92a inhibitor and treated with imatinib. Autophagic activity was studied using LC3B autophagy marker protein [24] (Fig. 6A). *MiR-92a-1-5p* inhibition increased LC3B variance of occupancy and signal intensity (Fig. 6B and C). In a second assay, chloroquine treatment (10 μM) was used to inhibit autophagic degradation by lysosomes (Fig. 6D). Similar results were obtained for LC3B variance of occupancy and signal intensity, however significance was not reached (Fig. 6E and F). Chloroquine treatment enabled the detection of larger structures corresponding to fused autophagosomes, allowing the quantification of autophagosome positive cells [25,26]. *MiR-92a-1-5p* inhibition increased the proportion of autophagosome positive cells independently of imatinib treatment, however a greater proportion was measured when combining the miR-92a inhibitor with imatinib. Moreover, chloroquine-treated *miR-92a-1-5p* overexpressing cells showed reduced p62 protein expression, another marker of autophagy (Supplementary Fig. 6).

3.7. MiR-92a-1-5p influences autophagy through BNIP3L downregulation

Next, we explored the role of *BNIP3L* in the increase of autophagy induced by *miR-92a-1-5p* inhibition. Immunofluorescence assays were performed on K562 cells transfected with miR-92a inhibitor and siRNAs targeting *BNIP3L*. In the absence of chloroquine treatment, no difference in LC3B signal distribution and intensity was measured throughout the different conditions (Supplementary Fig. 7A-C). BNIP3L variance of occupancy and signal intensity increased with *miR-92a-1-5p* inhibition and was restored to control levels following the transfection of BNIP3L siRNA (Supplementary Fig. 7D-F).

In cells treated with 10 μM of chloroquine, LC3B variance of occupancy and signal intensity increased significantly after transfection with miR-92a inhibitor alone, but no change was observed when combining the inhibitor with siRNAs targeting *BNIP3L* (Fig. 7A–C). The number of autophagosome positive cells which was increased by *miR-92a-1-5p* inhibition partially decreased when combined with siRNAs targeting *BNIP3L* (Fig. 7D). An increase in BNIP3L expression was observed after *miR-92a-1-5p* inhibition alone. SiRNAs reduced BNIP3L protein expression to levels lower than those observed in the control condition, independently of the miR-92a inhibitor (Fig. 7E). These observations were confirmed by image quantification (Fig. 7F and G).

4. Discussion

OncomiRs emerged as key contributors to leukemia development and progression, including CML [27]. They play critical roles in essential cellular processes such as proliferation, apoptosis and autophagy. This paper provides new evidence of the roles of *miR-92a-1-5p*, a member of the oncogenic *miR-17-92* cluster, in CML leukemogenesis.

Several miRNAs were found to be regulated by *BCR::ABL1*. Among them, expression of *miR-17-92* cluster was consistently downregulated by imatinib and siRNAs targeting *BCR::ABL1* [15]. This cluster acts as an oncogene in CML, regulating several pathways crucial for disease development and progression. It promotes cell proliferation, suppresses apoptosis, and enhances leukemogenesis through various mechanisms. The cluster members were studied individually or in combination. In K562, *miR-17-19b* overexpression enhanced cellular proliferation [15]. Transplantation of *BCR::ABL*-transduced hematopoietic cells from *miR-17-92* knock-out mice delayed leukemia development and improved survival. The proposed mechanism was that *miR-19b* targeted A20, inactivating NF-kB signaling which increased apoptosis and inhibited CML $CD34^{+}$ cell proliferation [16]. *MiR-17* and *miR-19a*

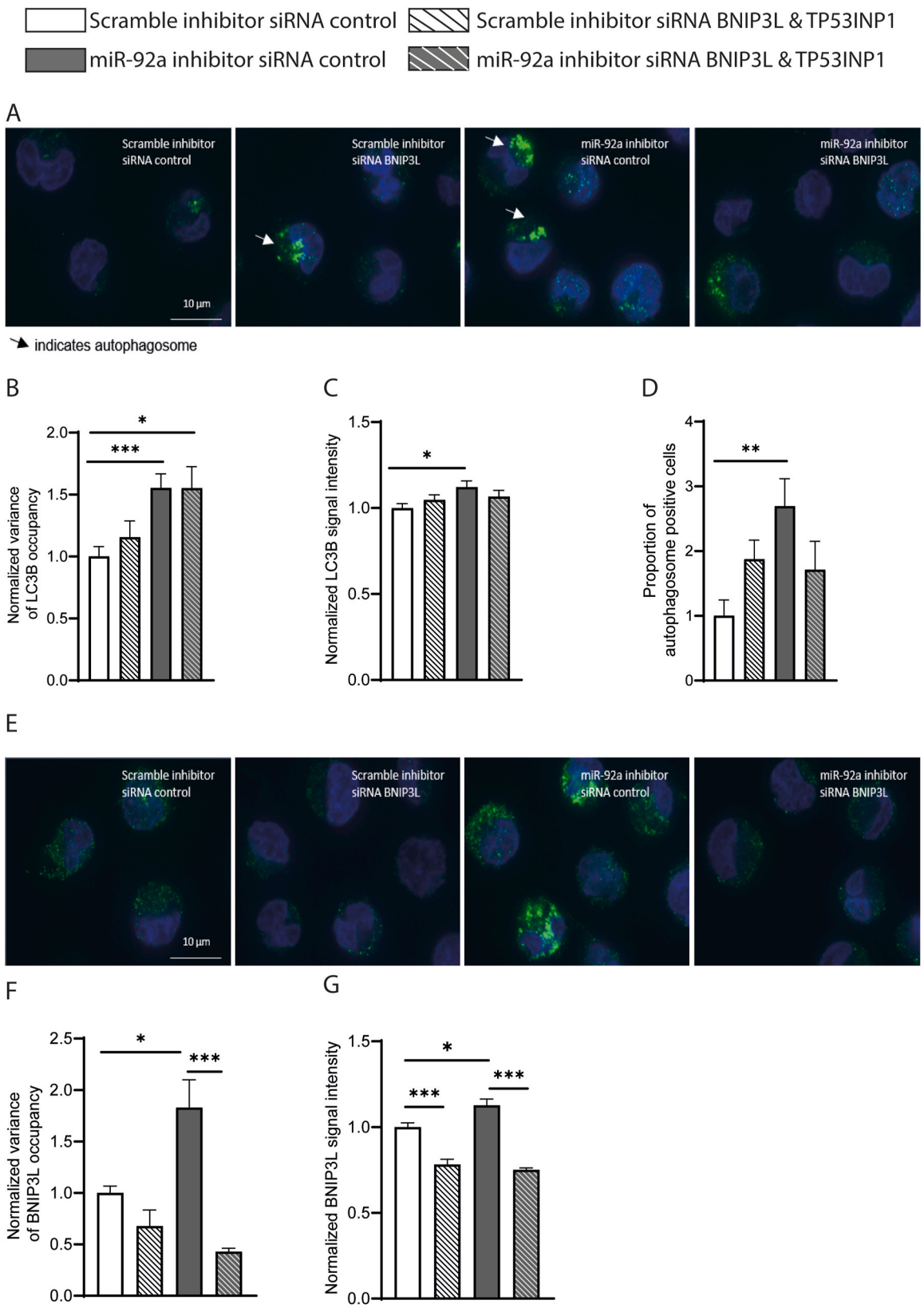

Fig. 7. Increase in autophagosome positive cells induced by *miR-92a-1-5p* inhibition is partially rescued after *BNIP3L* knockdown. K562 cells were transfected with scramble/miR-92a inhibitor and siRNA control/BNIP3L, and treated with 10 µM of chloroquine for 48h. Immunofluorescence assay was performed on LC3B (in green) and the nucleus was stained with Hoechst **(A)**. Images were analyzed with the Fiji software and variance of LC3B occupancy and LC3B signal intensity were quantified **(B & C)**. Autophagosome positive cells were quantified and normalized to the total number of cells **(D)**. On the same cells, immunofluorescence assay was performed on BNIP3L (in green) and the nucleus was stained with Hoechst **(E)**. Images were analyzed with the Fiji software and variance of BNIP3L occupancy and BNIP3L signal intensity were quantified **(F & G)**. For all panels, statistical comparison was performed with the One-way ANOVA test with or without Brown-Forsythe and Welch correction (N = 3; 5 images analyzed per experiment: *p < 0.05; **p < 0.01; ***p < 0.001).

directly regulate MAPK1, involved in MAPK signaling pathway in leucocytes [28,29]. Moreover, KRAS and RAF1 are predicted targets of *miR-19a* [30]. Unlike other miRNAs of the cluster, low expression levels of *miR-18a-5p* were measured in adriamycin resistant K562 cells, suggesting *miR-18a-5p* as a tumor suppressor in CML. *MiR-18a-5p* overexpression inhibited proliferation and induced apoptosis in resistant K562 cells. These studies confirm that each miRNA of the cluster regulates distinct tumor suppressor or growth-promoting genes by direct binding to 3′UTR of various targets.

Our study focused on the individual roles of *miR-92a-1-5p* in CML leukemogenesis. *MiR-92a-1-5p* was the most downregulated miRNA of the cluster in myeloid cell lines under TKI treatment and ranked among the ten most dysregulated miRNAs across all six cell lines (Supplementary Fig. 1). We first confirmed that imatinib treatment downregulated *miR-92a-1-5p* expression in two *BCR::ABL1* cell lines and CML patient samples. *MiR-92a-1-5p* inhibition alone was associated with a decreased cellular proliferation, while its overexpression promoted it. We observed that *miR-92a-1-5p* inhibition combined with imatinib treatment increased apoptosis, while its overexpression partially counteracted imatinib induced apoptosis. Moreover, in cells transfected with the miR-92a mimic, imatinib treatment led to a dose-dependent increase in *miR-92a-1-5p* expression, suggesting selective survival of highly transfected cells, while untransfected or poorly transfected cells were eliminated.

To further investigate *miR-92a-1-5p* oncogenic roles in CML, we explored its functions in *BCR::ABL1* positive cells through the identification of novel target genes. Our work focused on genes related to cellular proliferation, apoptosis and autophagy. Proliferation is the primary process driving myeloproliferative neoplasms, including CML [1]. Understanding the actors involved in cellular proliferation in CML is crucial for developing new treatments. Mechanisms of abnormal cellular proliferation described in CML involved *BCR::ABL1* signaling pathway, dysregulation of cell cycle regulators and inhibition of apoptosis [2]. We investigated whether the novel target genes *TP53INP1* and *BNIP3L*, played a role in the decreased proliferation induced by *miR-92a-1-5p* inhibition. Blocking both target genes counteracted the effect of *miR-92a-1-5p* inhibition on cell proliferation. *TP53INP1* inhibition alone, although in a lesser extent than inhibition of both target genes, is sufficient to rescue the effect of *miR-92a-1-5p* inhibition on cell proliferation however *BNIP3L* inhibition alone did not. *TP53INP1* is a tumor suppressor, involved in cell cycle arrest and whose expression is decreased in several cancers [22,23,31]. *TP53INP1* induced cell cycle arrest in G0-G1 phase in imatinib treated K562 cells, which was reduced upon direct inhibition of *TP53INP1* using siRNAs. This effect was replicated when *miR-92a-1-5p* was overexpressed, consequently reducing *TP53INP1* expression. *MiR-92a-1-5p* overexpression was reported to induce an increase in proliferation in cervical cancer by promoting cell cycle transition from the G1 to S phase, resulting in an enhanced invasion of cervical cancer cells [32]. We propose that *miR-92a-1-5p* promotes cell proliferation in *BCR::ABL1* positive cells, by targeting *TP53INP1* and thereby reducing cell cycle arrest.

Autophagy, a cellular degradation process, plays a dual role in cancer, by either promoting survival or contributing to cell death under different contexts [33]. All members of the *miR-17-92* cluster have been implicated in autophagy regulation [34]. We demonstrated that *miR-92a-1-5p* inhibition increased the abundance of autophagosomes, suggesting that this miRNA plays a regulatory role in autophagy. Inhibition of its target *BNIP3L (NIX)*, reversed the effect of *miR-92a-5p* inhibition on autophagosomes. *BNIP3L* (BCL2 Interacting Protein 3 Like) is a member of the BCL2 protein family involved in autophagy and apoptosis regulation through the forming of homodimers at the mitochondrial outer membrane following cellular stress [21]. The essential role of *BNIP3L* in erythrocyte differentiation and hematopoietic homeostasis has been demonstrated in mouse models, however little is known about its role in myeloid malignancies [35]. *BNIP3L* is recognized as a tumor suppressor with recent studies delving into its association with autophagy/mitophagy activity and cancer cell death [36]. The role of *BNIP3L* in CML is not known. *BCR::ABL1* leads to the upregulation of anti-apoptotic proteins like BCL2, contributing to leukemic cell survival. *BNIP3L* could counteract this survival advantage and induce apoptosis in leukemic cells. Our results suggest that promoting *BNIP3L*-mediated apoptosis or autophagy could improve the responses to TKI in CML.

Altogether, our data reveal that *miR-92a-1-5p* might play a key role in CML development and progression. *MiR-92a-1-5p* regulates cellular proliferation and cell cycle by targeting *TP53INP1* and autophagy by targeting *BNIP3L*. Inhibition of *miR-92a-1-5p* improves the anti-leukemic efficacy of imatinib suggesting that this miRNA can be a new therapeutic target in CML.

CRediT authorship contribution statement

Joanne Peters: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Emeline Bollaert:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anne-Sophie Cloos:** Writing – review & editing, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Melissa Claus:** Writing – review & editing, Methodology, Investigation, Data curation. **Ahmed Essaghir:** Writing – review & editing, Software, Data curation. **Sandrine Lenglez:** Writing – review & editing, Methodology. **Pascale Saussoy:** Writing – review & editing, Resources. **Guillaume Dachy:** Writing – review & editing, Software, Data curation. **Pierre Autin:** Writing – review & editing, Methodology, Data curation. **Jean-Baptiste Demoulin:** Writing – review & editing, Validation, Supervision. **Violaine Havelange:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Joanne Peters, Emeline Bollaert, Anne-Sophie Cloos and Melissa Claus performed experiments and collected data. Ahmed Essaghir and Pierre Autin analyzed the small RNA-sequencing data. Sandrine Lenglez helped with cloning, mutagenesis and luciferase assays. Pascale Saussoy provided patients samples. Guillaume Dachy and Pierre Autin analyzed the RNA-sequencing data. Jean-Baptiste Demoulin and Violaine Havelange supervised the study. Violaine Havelange and Joanne Peters designed experiments and wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ncrna.2025.05.008.

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