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Characterization of the impact of the *MYBBP1A* gene and rs3809849 on asparaginase sensitivity and cellular functions

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Aims: To investigate the role of *MYBBP1A* gene and rs3809849 in pancreatic cancer (PANC1) and lymphoblastic leukemia (NALM6) cell lines and their response to asparaginase treatment. **Materials & methods:** The authors applied CRISPR-Cas9 to produce *MYBBP1A* knock-out (KO) and rs3809849 knock-in (KI) cell lines. The authors also interrogated rs3809849's impact on PANC1 cells through allele-specific overexpression. **Results:** PANC1 *MYBBP1A* KO cells exhibited lower proliferation capacity ($p \leq 0.05$), higher asparaginase sensitivity ($p = 0.01$), reduced colony-forming potential ($p = 0.001$), cell cycle blockage in S phase, induction of apoptosis and remarkable morphology changes suggestive of an epithelial–mesenchymal transition. Overexpression of the wild-type (but not the mutant) allele of *MYBBP1A*-rs3809849 in PANC1 cells increased asparaginase sensitivity. NALM6 *MYBBP1A* KO displayed resistance to asparaginase ($p < 0.0001$), whereas no effect for rs3809849 KI was noted. **Conclusions:** *MYBBP1A* is important for regulating various cellular functions, and it plays, along with its rs3809849 polymorphism, a tissue-specific role in asparaginase treatment response.

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L-asparaginase (ASNase) is a key component in the treatment strategies of various leukemias and lymphomas, and it is universally incorporated into major childhood acute lymphoblastic leukemia (ALL) treatment protocols [1]. The authors previously reported the results of an exome-wide association study that identified several single-nucleotide polymorphisms (SNPs) associated with adverse drug reactions related to the administration of ASNase during ALL treatment [2]. Of those, rs3809849 in the *MYBBP1A* gene was associated with multiple major complications of ASNase exposure, among which the association with pancreatitis was replicated in an independent validation cohort [2].

MYBBP1A codes for the Myb-binding protein 1A (also known as p160), a 160 kDa protein that is expressed in all tissues, with a predominant localization in the nucleolus, where it is anchored by means of its interaction with RNA [3]. It is implicated in the stress response and carcinogenesis [4]. MYBBP1A is translocated from the nucleolus to the nucleoplasm in response to a decrease in the RNA content caused by stress signals [5,6]. The rs3809849 variant is located in the first exon of the *MYBBP1A* gene represented by a missense G to C substitution, which results in a glutamine to glutamic acid replacement (Gln8Glu) [2].

The MYBBP1A protein interacts physically with several nuclear transcription factors such as PGC-1α, NF-κB and p53 [7–12]. Several studies have described a functional role for *MYBBP1A* and its protein in essential biological functions such as cell division, cell proliferation, apoptosis and synthesis of ribosomal DNA [4,7,8,10,13–18], as well as displaying tumor suppressor activity [13]. When paired normal/tumor tissue samples were compared, a reduced *MYBBP1A* expression has been correlated to different malignancies (mainly of the pancreas, the liver and the kidneys), as well as to recurrent and metastatic tumors [8,19,20]. Collectively, these reports suggest that *MYBBP1A* has pleiotropic functions.

Acute pancreatitis is a common dose-limiting toxicity of ASNase that can occur in up to 18% of pediatric ALL patients [21–24]. The extent of pancreatitis severity can vary substantially between patients, ranging from mild and self-resolving symptoms to a severe systemic inflammatory response syndrome and failure of pancreatic function that can eventually precipitate acute or persistent diabetes mellitus [23–25]. Of note, recurrent pancreatitis can develop in up to 17% of patients that experience a first episode of acute pancreatitis [26], which has been associated with an increased risk of pancreatic cancer [27].

MYBBP1A gene expression has been shown to be enriched in endoderm during specific stages of endocrine pancreas development [28], and the level of its expression was found to be associated with the survival outcome in pancreatic cancer [29]. Moreover, pancreatic ductal adenocarcinoma cell lines, such as PANC1, are known to be hemizygous for *MYBBP1A*, and consequently express only 50% of the normal *MYBBP1A* levels [30]. A recent study found that hemizygous *MYBBP1A* loss in PANC1 promotes tumorigenesis, while homozygous loss of *MYBBP1A* is paradoxically associated with impaired cell growth and decreased tumorigenesis [30].

PANC1 pancreatic cancer cells have been shown to respond to ASNase treatment [31,32], and clinical trials are under way to explore the possibility of using ASNase for the treatment of pancreatic cancers [33]. Asparagine restriction in melanoma and pancreatic cancer cells activates the tyrosine kinase MAPK pathway, and MAPK inhibition was shown to ultimately reduce the expression of asparagine synthetase (ASNS), consequently sensitizing melanoma and pancreatic tumors to asparagine depletion and inhibiting their proliferation [34].

In this work, the authors further explore the link between *MYBBP1A* and rs3809849 as well as ASNase sensitivity in pancreatic cells as an initial effort toward understanding their role in pancreatitis. To that end, they applied CRISPR-Cas9 [35–37] and allele-specific overexpression techniques to study the functional impact of this gene and its polymorphism on the treatment response and cellular behavior of PANC1 cells. Moreover, given the previously identified association between rs3809849 and the survival outcome of ALL treatment [2], they also sought to investigate the impact of this polymorphism and the *MYBBP1A* gene on the treatment response of leukemic cells using the NALM6 cell line.

Materials & methods

Plasmids

LentiCas9-Blast (Addgene #52962, MA, USA) and pLentiGuide plasmids (Addgene #52963, MA, USA) were provided by the Gene Editing Platform of the CHU Sainte-Justine Research Center. LentiCas9-Blast and pLentiGuide are plasmids with lentiviral backbone that expresses human codon-optimized *Streptococcus pyogenes* Cas9 protein along with the blasticidin resistance gene and CRISPR chimeric RNA element with the puromycin resistance gene, respectively.

Cell lines

The PANC1 cell line was provided by Dr. Gerardo Ferbeyre's lab. All PANC1 derived cell lines (PANC1, PANC-Cas9, PANC1-Cas9-Δ*MYBBP1A*, PANC1-Cas9-*MYBBP1A*-rs3809849-MUT and PANC1-Cas9-*MYBBP1A*-rs3809849-WT) were maintained using Dulbecco's modified Eagle medium (DMEM)-based growth medium: DMEM (Wisent Inc., QC, Canada) + 10% fetal bovine serum (FBS; Sigma-Aldrich, Missouri, USA) + 1% Primocin® (InVivogen, CA, USA). The medium was changed every 3 to 4 days, and the cells were passed when reaching 90% confluence. The plating of PANC1 cell lines was done at 1×10^3 cells per well for the knock-out (KO), knock-in (KI) and overexpression experiments.

NALM6, NALM6-Cas9, NALM6-Cas9-Δ*MYBBP1A*, NALM6-Cas9-*MYBBP1A*-rs3809849-MUT and NALM6-Cas9-*MYBBP1A*-rs3809849-WT cell lines were cultured at a concentration of 1×10^6/ml in RPMI medium (Wisent Inc.) supplemented with 10% FBS + 1% Primocin and left to grow. The medium was changed every 2 to 3 days, and cells were split at a ratio of 1:2 to 1:3 as needed. For the ASNase sensitivity analysis, plating of NALM6 cell lines was done at 5×10^4 cells per well.

Generation of MYBBP1A knock-out & knock-in cell lines

KO and KI cell lines were developed by the Gene Editing Platform of the CHU Sainte-Justine Research Center (Supplemental Methods). All cell lines were incubated in 5% CO_2 at 37°C conditions. Puromycin 1 μg/ml was added to culture media to select for caspase-9 (Cas9)-expressing cells, and blasticidin (5 ng/ml; Wisent Inc.) was additionally used to further select for the cells expressing the sgRNA.

The production of Cas9 protein expressing wild-type PANC1 cells (PANC1-Cas9-WT) was confirmed by western blotting (Supplemental Figure S1A), and these cells were then used to produce the gene KO. The result of the mismatch assay indicated an approximately 30% efficiency within the cell population used for clonal selection (Supplemental Figure S1B). Clones were then probed for MYBBP1A protein expression by immunoblotting using anti-MYBBP1A antibodies (Supplemental Figure S1C). Four clones (PANC1-Cas9-Δ*MYBBP1A* clones 1, 2, 3 and 4) were selected for further analysis following FACS validation, as they exhibited an abolished MYBBP1A expression relative to PANC1-Cas9-WT (Supplemental Figure S1D).

Following the KO procedure in the NALM6 cell line, several clones without detectable expression of MYBBP1A compared with GAPDH were selected for subsequent characterization (Supplemental Figure S2A). The significant depletion of MYBBP1A expression compared with WT was further validated through FACS analysis (Supplemental Figure S2B).

Intriguingly, it was impossible to produce a stable *MYBBP1A*-rs3809849-MUT KI of the PANC1 cell line. While the PCR product following the nucleofection of PANC1-Cas9 cells with the RNA guide carrying the mutant form of the SNP showed a positive signal (thus indicating the presence of a significant percentage of a subpopulation that harbors the mutation), the clonal selection was unsuccessful in producing single-clone derived cultures of the *MYBBP1A*-rs3809849-MUT KI PANC1 cell line. The experiment was carried out three different times and approximately 200 clones were screened. This could imply that PANC1-Cas9 cells that did have a successful KI of the *MYBBP1A*-rs3809849-MUT had very poor survival and could not make it to the clonal selection step.

To verify whether the observed cell death following the KI experiment was related to a potentially deleterious SNP effect, or that it was arguably due to suboptimal experimental conditions, an identical experiment was performed using the same conditions, but with one modification. Accordingly, the donor sequence for KI was designed to introduce a Gln to His mutation at the same position of the protein (Supplemental Figure S3A) instead of the initially intended Gln to Glu substitution. By taking into consideration the fact that the production of this PANC1-*MYBBP1A*-8His cell line was readily achieved – and knowing that KI of rs3809849 mutation in NALM6 cells was successfully performed (Supplemental Figure S3C) – the data seem to suggest that the failure to produce the desired PANC1-*MYBBP1A*-rs3809849-MUT cell line was related to a SNP-specific damaging effect.

Generation of allele-specific MYBBP1A-rs3809849 overexpressing PANC1 cell lines

cDNA expression vectors for each of *MYBBP1A*-rs3809849-WT, *MYBBP1A*-rs3809849-MUT and a blank reference vector were designed and purchased through a commercial supplier (ABMgood, BC, Canada). Expression vectors used in this experiment were lentiviral plasmids that achieve target gene overexpression through a cytomegalovirus (CMV) promoter. To allow for the validation of transfection success and clonal selection, these vectors were designed so that the expression of the vector was coupled with a *GFP* reporter gene, as well as genes conferring resistance to kanamycin and puromycin. The general plasmid construct of these vectors is presented in Supplemental Figure S4.

These vectors were used to stably transfect the wild-type PANC1 cells followed by a series of selection and validation steps to successfully produce stable PANC1 cell lines overexpressing the *MYBBP1A*-rs3809849-WT, *MYBBP1A*-rs3809849-MUT or blank reference vector. The detailed methods are provided in the Supplemental Methods.

Drug sensitivity assay

In vitro sensitivity to *Escherichia coli* ASNase and vincristine (VCR) relative to *MYBBP1A* genotype/phenotype was assessed in PANC1 and NALM6 cells by calculating the drug concentration resulting in 50% inhibition of cell growth (IC_{50}).

Cells were plated in 96-well plates (1×10^3 cells per well) and treated with either ASNase (10 concentrations ranging from 0.0001 to 100 IU/ml) or VCR (7 concentrations ranging from 0.001 μm to 1000 μm). To establish the baseline proliferation capacity of each cell line, a positive control containing the cells and the culture medium – without any drug – was used to serve as the drug-free, 100% viability reference. A negative control containing

the culture medium and the drug – without any cells – was used to determine the cell-free background absorption. Following 48 h of incubation, 10 µl of WST-1 cell viability reagent (Roche Diagnostics, QC, Canada) was added to each well for a total reaction volume of 100 µl, and after 2 h, the absorbance was measured at a wavelength of 435 nm using ClarioStar microplate reader (BMG LABTECH, ON, Canada). The value corresponding to the reading of the negative control was subtracted from other readings to compensate for the background absorption. The viability of cells at each drug concentration was calculated as a percentage of the viability of the drug-free positive control. The experiment was performed in duplicate for each condition and repeated 6 independent times. IC_{50} values were estimated individually for each of the repetitions using GraphPad (Prism version 5.0) by fitting sigmoidal dose-response curves for each of the two drugs.

Proliferation assay

Measurement of the proliferation capacity was performed by plating PANC1 cells in a 96-well plate (1×10^3 cells per well) and observing their relative growth over 6 consecutive days. Briefly, on the day of plating, as well as on days 1, 2, 3, 4 and 5 post-plating, 10 µl of WST-1 cell viability reagent (Roche Diagnostics) was added to each well for a total reaction volume of 100 µl. After 2 h of incubation, the absorbance was measured at a wavelength of 435 nm using ClarioStar microplate reader, which directly correlates to the number of viable cells. The background absorbance was determined by adding the WST-1 reagent to wells that contained the culture medium without cells, and the value obtained was used to adjust for the background noise of the other wells by subtraction. Each condition was carried out in quadruplicate and repeated at least three independent times. The normalized data were then fitted into a non-linear regression curve using the exponential growth equation to allow for the evaluation of differences between the growth curves, and the calculation of doubling time (DT). The mean absorbance, along with the standard error, was calculated at each time point and the difference between each two cell lines (i.e., PANC1-WT vs PANC1-Cas9-WT, PANC1-Cas9-WT vs PANC1-Cas9-Δ*MYBBP1A*, PANC1-WT vs PANC1-Cas9-Δ*MYBBP1A*) was evaluated. The proliferation curves were produced using GraphPad (Prism version 5.0).

Colony-formation assay

The capacity of cells to produce colonies was evaluated in the presence and the absence of ASNase. For the drug-free condition, 500 cells were plated in a 6-well plate, covered with 2 ml of culture medium (DMEM + 10% FBS + primocin) and allowed to grow at 37°C in a humidified incubator for 2 weeks. For the drug-presence condition, 1000 cells were incubated for 48 h in a culture medium (DMEM + 10% FBS + primocin) containing ASNase (0.42 IU/ml; corresponding to the IC_{50} value determined in the previous experiment). Following the drug challenge, the medium was aspirated and replaced with ASNase-free culture medium. The cells were then incubated for 12 additional days (a total of 14 days from initial plating). At day 14, colony detection was performed by staining cells with methylene blue solution for at least 30 min. After staining, the plates were washed and air dried and colonies were counted manually against a bright background. The experiment was performed at least four-times for each condition and the difference in the number of colonies at day 14 between the two cell lines (in the presence and absence of ASNase) was evaluated.

Flow cytometry

The expression of *MYBBP1A* was quantified by immunofluroscence; 5×10^5 cells were re-suspended in 100 µl of 1 µg/ml of the primary antibody, then incubated for 30 min at room temperature and washed three-times by centrifugation at 400 × g for 5 min in ice-cold phosphate-buffered saline (PBS). Next, cells were re-suspended in 100 µl of fluorochrome-labeled secondary antibody diluted in 3% BSA/PBS and incubated for 30 min at 4°C in the dark, then washed three-times by centrifugation at 400 × g for 5 min in ice-cold PBS before checking their fluorescence levels.

The impact of genetic modification and ASNase exposure on the PANC1 cell cycle and apoptosis/necrosis was evaluated using a double staining flow cytometry approach. PANC1-Cas9-WT and PANC1-Cas9-Δ*MYBBP1A* cells were cultured in 6-well plates for 48 h, with and without ASNase, simultaneously. The cells were then harvested into a drug-free culture medium and prepared for the assay.

For the cell-cycle analysis, Click-iT™ EdU Alexa Fluor™ 647 Flow Cytometry Assay Kit (Invitrogen/Thermo Fisher Scientific, MA, USA) was used following the manufacturer's protocol, which employs 5′ethynyl-2′-deoxyuridine (EdU) and 7-amino-actinomycin D (7-AAD) staining. Briefly, cells were harvested and pulsed

with 10 µm EdU for 1.5 h and anti-EdU-antibody conjugated to Alexa Fluor 647 was used to stain EdU-positive cells, while 7-AAD was used to stain DNA. The percentage of cells in each phase was calculated following the display of the results as bivariate distribution of EdU content versus DNA content. The percentage of cells in the S phase was calculated by gating EdU-positive cells, while the percentage in the G0/G1 phase was calculated based on EdU-negative cells with low 7-AAD signal, and that of the G2/M phase was based on EdU-negative cells with high 7-AAD signal.

For the apoptosis analysis, cells were harvested and washed, and the dead and apoptotic cells were detected by Annexin V/propidium iodide (PI) solution staining using APC Annexin V Apoptosis Detection Kit with PI (BioLegend, CA, USA) following the manufacturer's instructions. Results were displayed as bivariate distribution of Annexin V staining versus PI staining.

All analyses were performed using FACSCanto II cytometer, and the results were processed using BD FACSDiva™ or Flowjo™ software. Experiments were performed on at least three independent cultures.

Quantitative PCR

Total RNA extracts were prepared in TransZol (Civic Bioscience, QC, Canada). Total RNA was reverse transcribed using 5X All-In-One RT MasterMix (Abmgood, British Columbia, Canada) on 2 µg of total RNA in 20 µl final volume according to the kit's instructions. Before proceeding to quantitative PCR (qPCR), reverse transcription products were diluted tenfold in RNAse free water. Real-time qPCR (RTqPCR) was performed using SYBR Green technologies (Roche Applied Science, CA, USA) as described previously [38]. The ΔΔCT method in a Lightcycler 480 (Roche LifeScience, QC, Canada) was used to perform the relative target-gene quantification of N-cadherin, vimentin and ZEB. The mRNA expressions were measured relative to the mRNAs of two housekeeping genes (*HMBS* and *TBP*). Experiments were performed on at least three independent cultures. Sequences of qPCR primers are provided in Supplemental Table S1.

Statistical analyses

All statistical analyses were done using the two-sided Student's *t*-test, except when comparing the global IC_{50} values between two fitted curves, which was done using the extra sum-of-squares F test. The p-value cutoff used to call statistical significance was $p < 0.05$ for all experiments and the numerical values were replaced by asterisks in the figures to simplify the presentation of the results (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Results

To study the role of *MYBBP1A* in the development of pancreatitis in response to leukemia treatment, the authors produced PANC1-Cas9-Δ*MYBBP1A* and NALM6-Cas9-Δ*MYBBP1A* by using the CRISPR-Cas9 approach. There was no significant difference between Cas9-expressing wild-type cells and their non-expressing counterparts in any of the experiments performed (data not shown). All results displayed in this work were based on the comparison between the cell lines that express Cas9 without the gene deletion (PANC1-Cas9-WT or NALM6-Cas9-WT) and with the deletion (PANC1-Cas9-Δ*MYBBP1A* or NALM6-Cas9-Δ*MYBBP1A*).

Impact of *MYBBP1A* on the PANC1 cell line

MYBBP1A gene expression significantly correlated to the *in vitro* sensitivity of PANC1 cells to ASNase ($p = 0.01$). KO cells were more sensitive to ASNase than PANC1-Cas9-WT cells (IC_{50} = 0.30 IU/ml vs 0.42 IU/ml, respectively; Figure 1A). Treatment with VCR did not show any significant difference ($p = 0.7$) relative to the presence or absence of the gene (Figure 1B). This selective increase in the sensitivity of PANC1-Cas9-Δ*MYBBP1A* to ASNase was maintained even after 96 h of incubation with the drug (Supplemental Figure S5).

The effect of *MYBBP1A* KO on cellular proliferation was evaluated in PANC1 cells over a period of 6 days and the results indicated a significant difference between the cellular growth curves ($p < 0.0001$), with KO cells demonstrating an overall slower proliferation process compared with their WT counterparts. Doubling time was longer for PANC1-Cas9-Δ*MYBBP1A* cells compared with PANC1-Cas9-WT cells (38.3 vs 34.0 h). The results demonstrated a divergence of the growth curves starting at day 2 post-plating, this difference became significant at day 4 ($p = 0.02$; Figure 2A) and continued to increase over time ($p = 0.001$ at day 5; Figure 2A).

The results of the clonogenic assay demonstrated that the disruption of *MYBBP1A* expression in PANC1 cells was associated with considerable changes in the characteristics of their colonies, along with a visible shift in their cellular morphology (Figure 2B). Notably, in the absence of drug exposure, KO PANC1 cells formed significantly

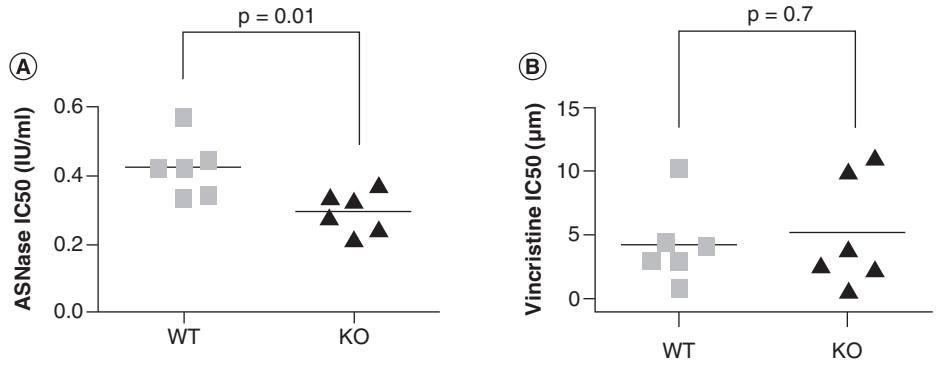

Figure 1. Evaluation of the *in vitro* sensitivity to asparaginase and vincristine in PANC1 cells in relation to *MYBBP1A* gene knock-out. The distribution of IC_{50} values of asparaginase or vincristine is plotted. IC_{50} values were calculated using WST-1 viability assay 48 h post-incubation with various concentrations of the drugs. The colored shapes represent independent IC_{50} values calculated for WT PANC1 cells (gray squares) or *MYBBP1A* knock-out PANC1 cells (black triangles). The vertical lines represent the mean IC_{50} value of each group. The p-value of the difference in drug sensitivity between the two cell lines is provided on top of each graph.
ASNase: Asparaginase; KO: Knock-out; WT: Wild-type.

less colonies than their WT counterparts (16% ± 4 mean reduction; p = 0.001; Figure 2C). Following a 2-day challenge with ASNase at a dose corresponding to the average IC_{50} value of the PANC1-Cas9-WT cells, a further decline in the number of colonies was proportionally observed in both cell lines (Figure 2C). Of note, the difference in clonogenic potential between the KO and WT cell lines that was initially observed in the absence of ASNase treatment continued to maintain its significance following drug exposure (13% ± 5 mean reduction; p = 0.02; Figure 2C).

Given the published reports showing that, when mitosis is abnormally prolonged, *MYBBP1A* plays a role in the activation of the intrinsic checkpoint machinery embedded in the cellular division process [39], the authors examined the impact of *MYBBP1A* KO on the progression of the cell cycle. The analysis results suggest that PANC1-Cas9-Δ*MYBBP1A* cells exhibit a distinct blockage in their cell cycle (Figure 3A), specifically at the S phase, as the percentage of cells in this phase was 12% higher when compared with PANC1-Cas9-WT cells (47.5% vs 35.7%, respectively). This increase was associated with a concomitant decrease in the number of cells in the G0/G1 phase (41.5% vs 49.9%) and G2/M phase (11% vs 14.4%). These differences between WT and KO cells were maintained after 48 h of ASNase exposure (15.3% vs 7.2% in the S phase, 78.6% vs 84.8% in the G0/G1 phase and 6.1% vs 8% in the G2/M phase; Figure 3A). However, following the treatment, a visible shift in the ratios of cells across the three phases of the cell cycle was observed in both cell lines. The most notable change was the increase in the number of cells in the G0/G1 phase at the expense of the two other phases.

The results also suggest that knocking-out *MYBBP1A* reduces PANC1 cells' viability (Figure 3B), since the proportion of healthy cells was 75.4% versus 91.5% of the total cell population in PANC1-Cas9-Δ*MYBBP1A* and PANC1-Cas9-WT cells, respectively. The Annexin V/7-AAD staining revealed that this difference stems from a substantial increase in the percentage of cells undergoing apoptosis, as 23% of PANC1-Cas9-Δ*MYBBP1A* cells versus 6.8% of PANC1-Cas9-WT cells were apoptotic. Of these, 19.6% and 3.9%, respectively, were early apoptotic cells, while 3.4% and 2.9%, respectively, were late apoptotic cells. The percentage of cells undergoing necrosis did not change between the edited and wild-type cell lines (1.7% vs 1.6%, respectively). Similar results were obtained following 48 h of exposure to ASNase (Figure 3B).

When performing the colony-formation assay, the authors observed that the colonies formed out of PANC1-Cas9-Δ*MYBBP1A* cells were visibly less dense and more diffused compared with the colonies derived from the PANC1-Cas9-WT control cell line. In fact, the lack of *MYBBP1A* gene expression seems to have provoked a distinctive change in cellular morphology, reminiscent of the ones seen during an epithelial–mesenchymal transition (EMT). Accordingly, the authors measured the levels of N-cadherin, vimentin and ZEB, three markers associated with EMT. The results suggested a general increase in the relative mRNA expression levels for all three markers (Figure 4A), with vimentin showing a significant, threefold increase in PANC1-Cas9-Δ*MYBBP1A* cells compared with PANC1-Cas9-WT cells in the absence of ASNase exposure (p = 0.05). Following 48 h of incubation with

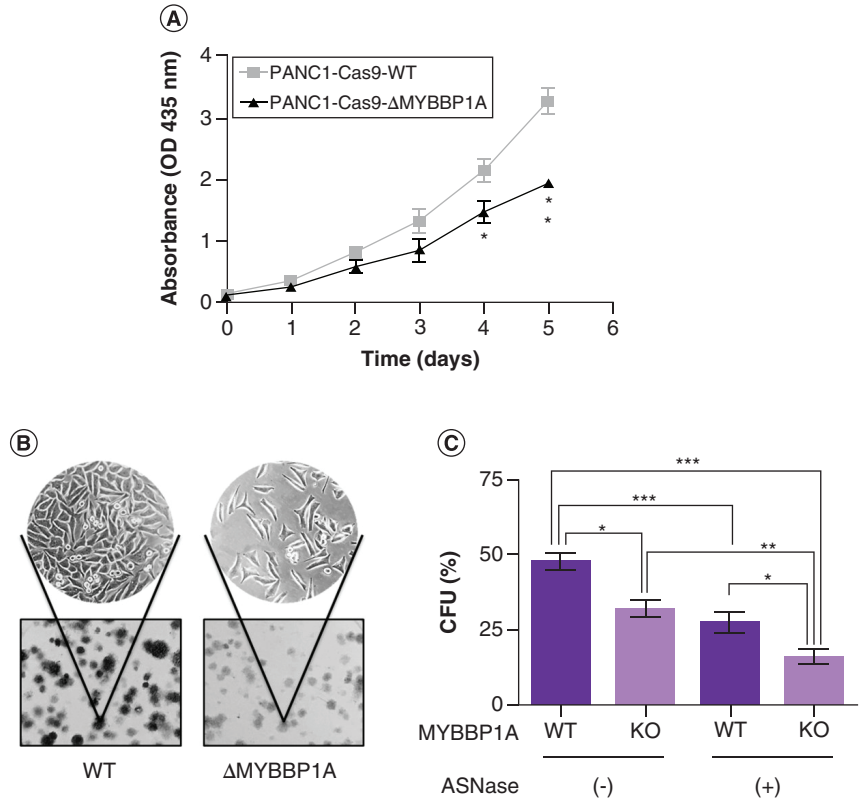

Figure 2. Effect of *MYBBP1A* gene knock-out in PANC1 cells on their cellular proliferation rate (upper panel) and their colony-forming capacity in relation to asparaginase (ASNase) exposure (lower panel). Upper panel: **(A)** The proliferation of *MYBBP1A* gene knock-out PANC1-Cas9 cells (PANC1-Cas9-Δ*MYBBP1A*, black triangles) and wild-type PANC1-Cas9 control (PANC1-Cas9-WT, gray squares) was measured using the WST-1 cell proliferation assay. Absorbance was measured at 435 nm on days 0, 1, 2, 3, 4 and 5, post-plating. The quantitative data shown are the mean absorbance ± SEM from at least 3 separate experiments per condition.
Lower panel: Cells were incubated in either ASNase-containing or ASNase-free culture medium for 48 h; then all media were replaced by a drug-free medium and the number of colonies formed after a total of 14 days of plating was counted manually. **(B)** A macroscopic view of a representative plate showing the difference in the number and density of colonies formed starting from the same quantity of PANC1 cells (with and without *MYBBP1A* gene deletion) along with a microscopic magnification of one of the colonies to demonstrate the change in the morphology of the cells and intercellular spacing. **(C)** The quantification of the colony-forming unit data showing the average number of colonies from all experiments along with the error bars is provided. Note that the difference in clonogenic capacity was already visible even without ASNase treatment (n = 159 ± 13.6 SEM for KO vs n = 239 ± 14.2 SEM for WT) and was maintained following a 48-h challenge with the drug (n = 77 ± 2.5 SEM for KO vs n = 226 ± 1.5 SEM for WT).
ASNase: Asparaginase; CFU: Colony-forming unit; KO: Knock-out; OD: Optical density; SEM: Standard error of the mean; WT: Wild-type.

ASNase, the level of vimentin surged significantly in PANC1-Cas9-WT (sixfold; p = 0.0007), but not in PANC1-Cas9-Δ*MYBBP1A*, when compared with the respective untreated counterpart (1.2-fold; p = 0.6; Figure 4B).

In view of the perplexing observation regarding the inability to produce a viable *MYBBP1A*-rs3809849-MUT KI in the PANC1 cell line (see Methods section for details), the authors sought to obtain more information on the specific role that this SNP plays in pancreatic cells – particularly, its influence on their sensitivity to ASNase. To do so, they performed complementation studies contingent on allele-specific overexpression. Accordingly, differential overexpression of the two *MYBBP1A*-rs3809849 alleles was achieved through transfection of PANC1 cells with specifically designed expression vectors. Successful transfection was confirmed by observing cell growth in a selection medium containing puromycin (Supplemental Figure S6A), as well as through green fluorescent protein (GFP) expression using microscopy and flow cytometry (Supplemental Figure S6B). The expression of the target allele was confirmed through allele-specific PCR (Supplemental Figure S6C). Each single-clone-derived cell culture was tested by PCR to verify the presence of the transfection vector, and the overexpression of *MYBBP1A* was quantified by RTqPCR (Supplemental Figure S6D). Collectively, these results indicated that stable PANC1 cell

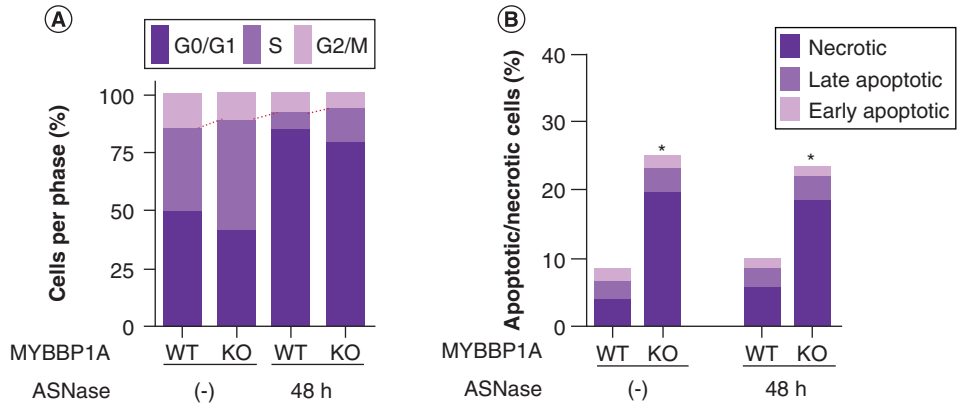

Figure 3. Impact of *MYBBP1A* gene deletion on PANC1 cellular functions. (A) Cell cycle. Percentage of cells in each of the 3 main cell-cycle phases. Cells were incubated for 48 h in culture medium without (-) or with (48 h) asparaginase, respectively. Each bar represents 100% of cells in each condition indicated at the bottom of the graph. Colored sections of the bar represent the percentage of cells per respective phase as indicated on the top of the graph. The red-dotted line highlights the additive trend of reduction in percentage of cells entering the G2/M phase. **(B)** Apoptosis/necrosis, and their response to ASNase exposure. Percentage of early apoptotic, late apoptotic and necrotic cells. Cells were incubated for 48 h in culture medium without ASNase (-), or with ASNase (48 h). Each bar represents the fraction of unhealthy cells in each condition as indicated at the bottom of the graph. Colored sections of the bar represent the percentage of cells in each category as indicated on the top of the graph.
*Significant difference between the treated cells and their untreated counterparts.
ASNase: Asparaginase; KO: PANC1-Cas9-Δ*MYBBP1A*; WT: PANC1-Cas9-WT.

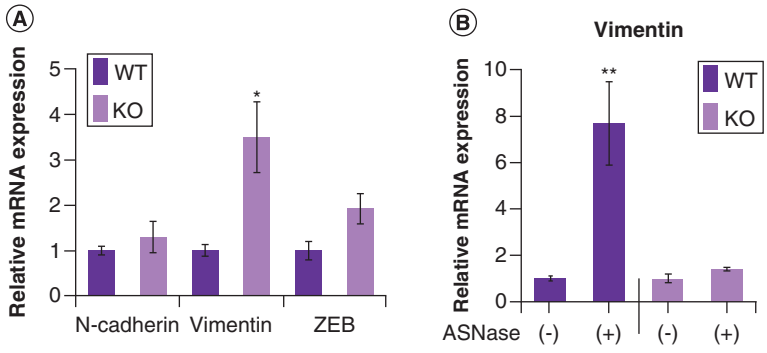

Figure 4. Relative expression of markers associated with epithelial–mesenchymal transition in PANC1 cells in response to *MYBBP1A* gene deletion and asparaginase exposure. (A) *MYBBP1A* gene deletion. Expression of N-cadherin, vimentin and ZEB without asparaginase (ASNase) exposure presented as fold-change in KO versus WT. A significant difference in vimentin's level between the two cell lines prior to drug exposure is represented by the asterisks on top of the bar. **(B)** Asparaginase exposure. Colored bars represent the relative mRNA expression levels of epithelial–mesenchymal transition markers in PANC1-Cas9-WT wild-type cells (WT) and PANC1-Cas9-ΔMYBBP1A edited cells (KO) following 48 h of incubation. Impact of a 48-h treatment with ASNase on the levels of expression of vimentin in both cell lines.
*p-value of the difference observed within the same cell line in the presence and absence of ASNase.
ASNase: Asparaginase; KO: PANC1-Cas9-Δ*MYBBP1A*; WT: PANC1-Cas9-WT.

lines overexpressing the respective vectors for *MYBBP1A*-rs3809849-WT, *MYBBP1A*-rs3809849-MUT and the blank reference were successfully obtained by transfection.

The result of the drug sensitivity analysis indicated that the overexpression of *MYBBP1A* in the WT form (*MYBBP1A*-rs3809849-WT) rendered PANC1 cells more vulnerable to ASNase, as reflected by a reduced viability of these cells following 48 h of drug challenge. The comparison of IC_{50} data demonstrated a higher ASNase sensitivity for *MYBBP1A*-rs3809849-WT compared with PANC1 cells overexpressing the blank reference vector (IC_{50} = 0.39 IU/ml vs 0.66 IU/ml, respectively; p = 0.03; Figure 5). This difference was also visible when comparing *MYBBP1A*-rs3809849-WT with *MYBBP1A*-rs3809849-MUT, but it did not reach the statistical threshold of significance (IC_{50} = 0.39 IU/ml vs 0.57 IU/ml, respectively; p = 0.09; Figure 5). However, the overexpression

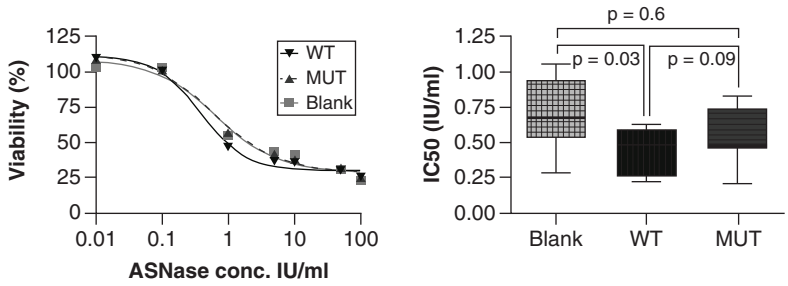

Figure 5. Influence of *MYBBP1A*-rs3809849 (wild-type vs mutant alleles) overexpression on asparaginase (ASNase) response in the PANC1 cell line. Comparison of IC_{50} values between PANC1 cells overexpressing the *MYBBP1A* gene with rs3809849 in the wild-type form (WT, GG) and mutant form (MUT, CC), compared with PANC1 cells overexpressing the empty vector (blank) following 2 days of treatment with varying concentrations of ASNase. ASNase: Asparaginase; MUT: Mutant allele; WT: Wild-type allele.

of *MYBBP1A*-rs3809849-MUT was not associated with a significant difference in the sensitivity of PANC1 cells compared with the blank control (Figure 5).

Impact of *MYBBP1A* on NALM6 cell line sensitivity

The NALM6-Cas9-Δ*MYBBP1A* cells were significantly more resistant to ASNase compared with the wild-type NALM6-Cas9-WT cells, as reflected by the 2.8-fold increase in the IC_{50} value (IC_{50} = 0.48 IU/ml vs 0.17 IU/ml, respectively; $p < 0.0001$; Figure 6A). This effect was drug-specific, since the control analysis did not demonstrate a significant difference in KO cells' sensitivity to VCR ($p > 0.05$; Figure 6A). The results from the KI cell line, however, did not show any difference compared with the WT cell line for either of the drugs, as shown in Figure 6B.

Discussion

The *MYBBP1A* gene has been linked to several essential biological functions spanning cell proliferation, cell division, apoptosis and synthesis of ribosomal DNA, among others [4,7,8,10,13–18]. It has been proven essential for normal cellular functions and is highly evolutionarily conserved among species [40], given that the MYBBP1A protein plays a key role at the interface between the transcription and processing functions [41].

Recently, the results of an exome-wide association study detected an association between the mutant allele of rs3809849 polymorphism located in the *MYBBP1A* gene and an increased risk of multiple ASNase-related complications, as well as poor survival outcome in pediatric ALL patients. Notably, its association with pancreas toxicity was replicated in an independent validation cohort [2]. In this follow-up study, the authors explored the role that the *MYBBP1A* gene plays in modulating ASNase sensitivity in pancreatic and leukemic cells using PANC1 and NALM6 cell lines, respectively. They also examined the impact of *MYBBP1A* gene deletion on various cellular functions of PANC1 cells, both in the absence and in the presence of ASNase exposure. Moreover, they attempted to characterize the specific interaction between rs3809849 and ASNase treatment in these two cells lines.

The *MYBBP1A* KO PANC1 cells demonstrated a 30% increase in sensitivity to ASNase. Importantly, this difference in sensitivity was unique to ASNase, since the deletion of the *MYBBP1A* gene did not affect the *in vitro* sensitivity to VCR, which exerts its function through a mechanism distinct from that of ASNase. Modulation of MYBBP1A protein levels has been linked to DNA damage caused by treatment of tumor cells with etoposide, whose antineoplastic mechanism of action as an antimetabolite resembles that of ASNase [4]. In contrast to PANC1, the *MYBBP1A* KO NALM6 leukemia cell line showed a higher resistance to ASNase of almost threefold, further confirming the presence of a selective interaction between ASNase and *MYBBP1A,* but also suggesting that *MYBBP1A* might have a tissue-dependent effect. In fact, this observation of an increased IC_{50} upon *MYBBP1A* KO in this leukemia cell line is consistent with results from another study that reported a strong negative correlation between the expression level of this gene in lymphoblastoid cell lines and IC_{50} values of gemcitabine and cytosine arabinoside, both also antimetabolites [42].

MYBBP1A KO PANC1 cells had a longer doubling time compared with the WT, a difference that was significant at day 4 of plating and beyond. This is in line with other studies showing a threefold reduction in cellular growth upon *MYBBP1A* gene silencing in pancreatic ductal adenocarcinoma cells (PANC1, ASPC1 and BXPC3) [30], as well as in other cell lines such as head and neck squamous cell carcinoma [8] and HeLa cells [13,41]. Moreover,

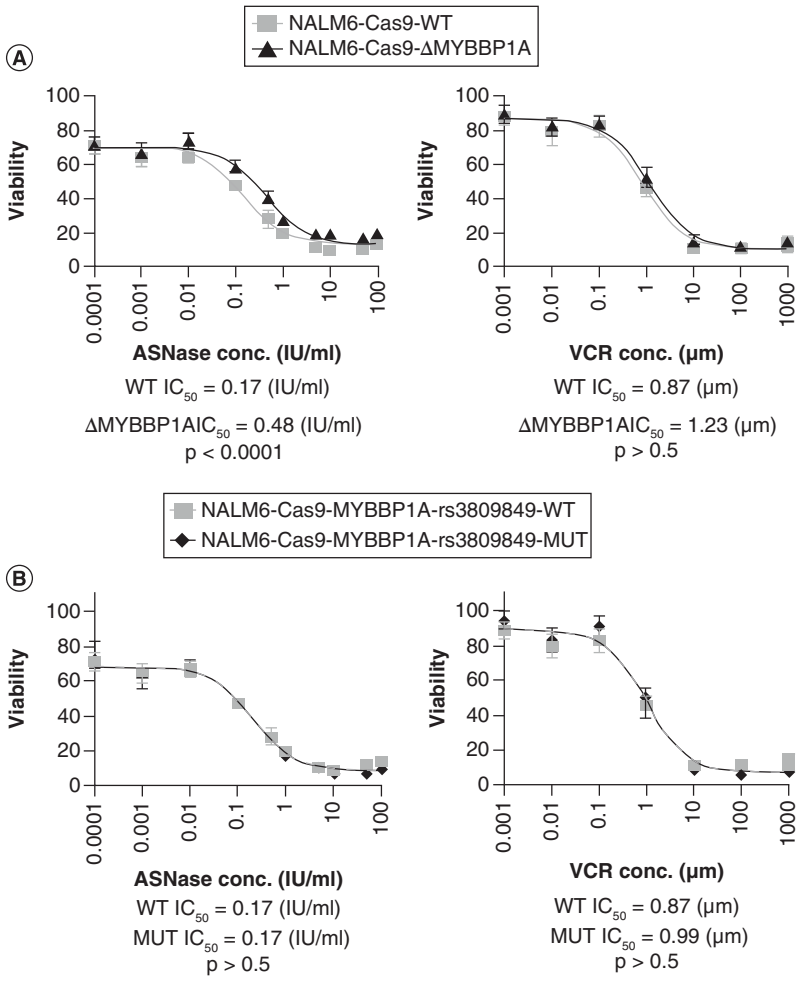

Figure 6. Influence of *MYBBP1A* genetics on asparaginase response in the NALM6 cell line. Comparison of IC_{50} curves between NALM6-Cas9-WT and **(A)** NALM6-Cas9-Δ*MYBBP1A* or **(B)** NALM6-Cas9-*MYBBP1A*-rs3809849-MUT. Data are provided for both asparaginase and vincristine following 2 days of treatment with varying concentrations of the drugs. The p-values represent the significance of the differences between the means and are provided at the bottom of each graph.
ASNase: Asparaginase; MUt: Mutant allele; VCR: Vincristine; WT: Wild-type allele.

the colony-formation assay demonstrated a significant reduction in the number of colonies in *MYBBP1A* KO PANC1 cells, which further implies that abolishing *MYBBP1A* expression in PANC1 cells reduces their capacity to replicate, possibly rendering them less capable of compensating for the damage caused by various stressors. This effect was maintained when cells were challenged with ASNase. The reduction in the clonogenic potential upon suppressing *MYBBP1A* expression is consistent with the results found in the HS766T pancreatic cell line [30] and in hepatocellular carcinoma cells [20]. However, it is in contrast with findings from other studies that reported an increase in the clonogenic potential of other cell lines upon *MYBBP1A* downregulation, such as in the Ras-transformed NIH3T3 mouse embryonic fibroblast cell line [13], breast cancer cells [14] and a specific set of renal cancer cells [19]. Such a discrepancy could be explained by the growing body of evidence suggesting that the role of the *MYBBP1A* gene in cellular viability and proliferation is tissue-specific and context-dependent. Opposing effects of its dysregulation were reported in various cell lines and even at different stages of tumor development in the same cell line [8,13]. Likewise, low levels of expression of this gene were associated with lower survival in pancreatic cancer, while in renal cancer, melanoma and thyroid cancer, higher levels of its expression showed a worsening prognosis (Supplemental Figure S7) [8,19,20,29].

MYBBP1A is an important component of a sensor-like structure that connects the machinery of the cell cycle to the energy of the cell, and several studies have linked *MYBBP1A* to cell cycle control and mitosis [13,16,39]. For

instance, silencing the *MYBBP1A* gene in HeLa cells was shown to induce the activation of genes that inhibit growth and the suppression of genes involved in DNA repair, ultimately provoking a cell cycle arrest [13]. Moreover, reduced expression of *MYBBP1A* was shown to delay the progression of mitosis and to result in failures in the assembly and stability of the mitotic spindle [16]. It is also involved in the activation of the checkpoint machinery when mitosis is abnormally prolonged [39]. Indeed, the present cell cycle analysis shows that knocking-out *MYBBP1A* in PANC1 cells results in a blockage at the S/G2-M checkpoint, suggesting slower growth and a reduced capacity to complete the processes of cytokinesis and spindle formation needed to enter mitosis. This could explain the observed reduction in the proliferation rate of PANC1 KO cells compared with WT cells. In concordance with what is already known about the impact of ASNase on the cell cycle [43], the results of the current study demonstrate that ASNase exposure blocks the PANC1 cells in the G0-G1/S checkpoint. This is plausibly due to the incapacity of cells to move forward with the protein synthesis process required for cytokinesis as a result of depletion of the amino acid asparagine (and probably glutamine) [44] caused by the mechanism of action of the drug [1]. Interestingly, the effect of *MYBBP1A* KO on the S phase of the cell cycle was maintained upon ASNase exposure, consequently resulting in a twofold increase in the percentage of PANC1-Cas9-Δ*MYBBP1A* cells blocked in the S phase compared with PANC1-Cas9-WT.

Moreover, this experiment provides evidence on the involvement of the MYBBP1A protein in PANC1 cells' apoptosis. Knocking-out *MYBBP1A* in these cells resulted in a significant reduction in the percentage of healthy cells with a concomitant increase in the percentage of cells undergoing apoptosis (but not necrosis), an observation that is consistent with results from studies in HeLa cells previously reported by others [13]. Interestingly, this difference was maintained between the WT and KO cell lines following 48 h of incubation. One of the suggested mechanisms that would lead to apoptosis [45] is modulation of p53 activity. Nevertheless, the mRNA expression analysis of p53 did not show a significant difference between KO and WT PANC1 cells in the absence of treatment, or after 48 h of treatment with ASNase (Supplemental Figure S8). This is in concordance with the studies suggesting that the interaction between *MYBBP1A* and p53 is mediated at the protein level, including epigenetic post-translational modifications [7,15,18].

Taking all the abovementioned observations into account, the authors hypothesize that the impact of *MYBBP1A* gene deletion on PANC1 cells is a result of a specific cell cycle blockage in the S phase, along with an induction of apoptosis, thus reducing the proliferation rate and the clonogenic potential of the KO cells. Moreover, these effects were maintained following treatment with ASNase.

Of note, *MYBBP1A* gene knock-down has been shown to impact the cellular morphology of HeLa cells, which displayed an abnormal, flattened and enlarged morphology upon gene depletion [41]. Visual examination of the cellular morphology of PANC1 cells following the KO of *MYBBP1A* also revealed significant changes in their morphology, as they became more spindle-shaped and distant from each other. These features seem to reflect an EMT process, according to which cells lose their polarity and eventually acquire a fibroblast-like phenotype. Specifically, these cells seem to lose cell–cell adhesion and show more intercellular spacing, a process that is often associated with acquiring migratory and invasive properties [46,47]. Upon measuring the relative mRNA expression levels of markers associated with the EMT process in *MYBBP1A* KO and WT PANC1 cells, the authors observed a general increase in the levels of the tested markers, with vimentin, a mesenchymal phenotypic marker, showing a significant, 3.5-fold increase in expression. Following 48 h of incubation with ASNase, the levels of vimentin surged significantly in PANC1-Cas9-WT cells, but not in PANC1-Cas9-Δ*MYBBP1A* cells, further supporting the assumption that the latters were already at a mesenchymal state prior to being exposed to the treatment. This EMT process, and a very similar phenotype to the one observed in this study, were documented in PANC1 cells following exposure to different stressors such as TGF-β [48] or incubation in a hypoxic environment – which was shown to be mediated by NF-κB activation [46].

Several findings in the literature support the hypothesis that abolishing *MYBBP1A* expression can induce EMT/inflammation. For example, *MYBBP1A* is recognized as a transcriptional co-repressor of NF-κB, and the activation of the NF-κB pathway is linked to the development of acute pancreatitis [49], as well as to EMT [50,51]. In fact, the EMT/inflammation/metastasis pathway has lately been investigated as a potential therapeutic target in a murine pancreatic cancer model, whereby the suppression of EMT using nimbolide was shown to successfully hamper metastasis [52].

Regarding the role of rs3809849, while the production of a viable *MYBBP1A*-rs3809849-MUT KI PANC1 cell line was not feasible, the results from the overexpression experiment indicate that upregulating the expression of the WT form of *MYBBP1A*-rs3809849 (but not the mutant allele) was associated with a 40% increase in the

sensitivity to ASNase, plausibly implying an allele-dependent effect in PANC1 cells. Nonetheless, the results from the KI experiment in NALM6 cells did not show an impact for the mutant form on the sensitivity to ASNase, which further argues in favor of a tissue-dependent effect of *MYBBP1A*-rs3809849. In addition, it is pertinent to mention that this polymorphism is in linkage disequilibrium with rs3760194 in the promoter region of the *MYBBP1A* gene and that they both act as strong expression quantitative trait loci for *MYBBP1A* and *GGT6* genes in various tissues, according to the Genotype-Tissue Expression (GTEx) database [53]. In fact, *GGT6* gene could be of particular interest in the context of pancreas toxicity (Supplemental Figure S9) [53], since it belongs to the human gamma-glutamyltransferase gene family that also includes *GGT1* [54], whose polymorphisms have been associated with acute and chronic pancreatitis [55,56].

The current observations regarding the impact of *MYBBP1A* KO on PANC1 cells are in concordance with similar results reported in the literature showing that disturbing this gene's expression in PANC1 cell line results in significant alterations in cellular growth and migratory properties [30,57]. ASNase-induced pancreatic injury was suggested to be a result of imbalanced plasma amino acid levels [58]. Understanding how pharmacogenetics influence the response of different cell types to ASNase at a molecular level not only holds the potential to reduce the risk of ASNase-induced toxicities and to improve ALL treatment outcome, but also can help refine treatment strategies for pancreatic cancers in which asparagine and/or glutamine depletion might be indicated [59]. Indeed, the influence of these two amino acids on the survival, growth and metastatic potential of tumor cells via metabolic adaption mechanisms has gained more attention over the past few years [34,60]. Moreover, combinations of erythrocyte-encapsulated ASNase with other chemotherapeutic agents (some of which having previously demonstrated *MYBBP1A*-influenced activity) [2,42] were investigated as a second-line treatment for advanced pancreatic cancer in clinical trials, and the results demonstrated an improved disease control rate overall, along with encouraging survival benefits for certain subgroups [33,61].

Finally, the authors acknowledge that this work has certain limitations. While it demonstrates a functional implication of the *MYBBP1A* gene in PANC1 cells' response to ASNase as a proxy for pancreatic cells, it is possible that the malignant transformation of PANC1 cells could have influenced their drug sensitivity and/or the expression profiles of other important genes involved in the pathway of the studied response. Accordingly, pharmacogenetic studies of ASNase response in another cell line more representative of 'normal' human pancreatic cells, such as HPNE, as well as in animal models of pancreatitis, would be of great value. Likewise, despite the results from the allele-specific overexpression experiment suggesting an influence of rs3809849 on ASNase sensitivity in PANC1 cells, this finding should be interpreted with caution, given the inconclusive results from the KI experiments and the observed absence of such a role in NALM6 cells. Moreover, further experiments are required to provide a mechanistic model that can explain the involvement of *MYBBP1A* in modulating the risk of ASNase-induced acute pancreatitis at a molecular level. In the same context, studies designed to identify drugs that can target *MYBBP1A* gene expression or its protein's function (such as kinases) could prove beneficial in understanding the mechanisms of *MYBBP1A* regulation and exploiting its clinical potential.

Conclusion

The results of this study suggest that *MYBBP1A* KO PANC1 cells are essentially unstable and therefore undergo a specific cell cycle arrest and subsequent spontaneous apoptosis, along with the activation of an EMT program, an effect that is more visible following their exposure to ASNase. This elucidates that the *MYBBP1A* gene plays a critical role in the proliferation of PANC1 cells and provides further evidence in relation to its implication in the modulation of the sensitivity of pancreatic cells to ASNase treatment. *MYBBP1A*-rs3809849 polymorphism seems to play a role in the survival of PANC1 cells and their response to treatment with ASNase, but no such role was observed in NALM6 cells. Moreover, contrary to PANC1 cells, knocking-out *MYBBP1A* in the NALM6 cell line was associated with an increased resistance to ASNase, therefore providing further evidence to support a cell-type dependent effect for the *MYBBP1A* gene and its rs3809849 polymorphism.

Author contributions

M Krajinovic and R Abaji designed the study; R Abaji, V Roux, IR Yssaad, P Kalegari, V Gagné and R Gioia performed the experiments; R Abaji performed the data analysis; C Beauséjour supervised the gene editing process in the platform; G Ferbeyre provided the expertise and supervision; M Krajinovic provided the funding; R Abaji drafted the article; all authors revised the manuscript.

Summary points

- L-asparaginase (ASNase) is a key component in the treatment strategies of various leukemias and lymphomas and is being investigated as a therapeutic agent for several types of cancer.
- rs3809849 in the *MYBBP1A* gene has been associated with multiple major complications related to treatment with ASNase, notably with pancreatitis.
- Several essential biological functions were linked to the *MYBBP1A* gene, including cell proliferation, cell division, apoptosis and ribosomal DNA synthesis, among others.
- *MYBBP1A* encodes for the Myb-binding protein 1A, which is implicated in the stress response and carcinogenesis, as it interacts with several nuclear transcription factors such as PGC-1α, NF-κB and p53.
- Knocking-out *MYBBP1A* in the PANC1 cell line resulted in lower proliferation capacity ($p \leq 0.05$), higher asparaginase sensitivity ($p = 0.01$), reduced colony-forming potential ($p = 0.001$), cell cycle blockage in S phase and induction of apoptosis, in addition to remarkable morphology changes compatible with an epithelial–mesenchymal transition.
- The overexpression of the wild-type, but not the mutant, allele of *MYBBP1A-rs3809849* in PANC1 cells was associated with an increased asparaginase sensitivity.
- NALM6 *MYBBP1A* knock-out cells exhibited an increased resistance to asparaginase ($p < 0.0001$), whereas no effect for rs3809849 knock-in was noted.
- These results suggest that the *MYBBP1A* gene and its rs3809849 polymorphism affect the cellular functions of PANC1 cells, and they seem to play a tissue-specific role in the response of PANC1 and NALM6 cells to treatment with ASNase.

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Supplementary material

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/pgs-2022-0010

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

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