



Original article

MG132 inhibits the expression of PBX3 through miRNAs by targeting Argonaute2 in hepatoma cells

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ABSTRACT

Cancer stem cells play important roles in the development of tumors also are important targets to therapy of cancer. Former researches had confirmed the pre-leukemia transcription factor 3 (PBX3) was involved in maintaining the characteristics of liver cancer stem cell. We found that PBX3 is an extremely unstable protein with a short half-life in hepatocellular carcinoma cells. Unstable proteins are believed to be susceptible to degradation by ubiquitin-proteasome system. However, when we treated hepatoma cells using the proteasome inhibitor MG132, found the levels of PBX3 protein and mRNA were significantly downregulated, suggesting that PBX3 protein is not degraded by the ubiquitin-proteasome system. Our study aims to investigate the mechanism of MG132 regulation of PBX3. We observed that the levels of miR-424, let-7c, miR-222, miR-200b were upregulated when hepatoma cells were treated with MG132, and this increase was negatively correlated with the levels of PBX3. Using the miRWalk algorithm, previous studies have predicted that these miRNAs target the PBX3 gene. Thus, we investigated the mechanism by which the proteasome inhibitor MG132 regulates these miRNAs. It has been reported that the Argonaute2 protein is an important component of the RNA-induced silencing complex (RISC), and it can regulate the levels of certain miRNAs. Consequently, we also investigated whether the proteasome inhibitor regulates related miRNAs by stabilizing Argonaute2. Using co-infection, co-immunoprecipitation (Co-IP), and western blot assays, we found that MG132 stabilizes the expression of the Argonaute2 protein by inhibiting its degradation via the ubiquitin-proteasome pathway. In summary, the PBX3 protein, which is closely linked to the stemness of hepatoma cells, does not undergo degradation by the ubiquitin-proteasome system (UPM).

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1. Introduction

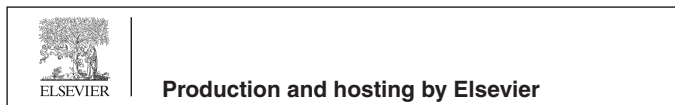
Hepatocellular carcinomas (HCC) are one of the most common malignant tumors reported in clinical practice. In terms of worldwide incidence and mortality, HCC ranks sixth and third, respectively, among all cancers. The current incidence rate is over 600,000 per year, and the incidence rates are increasing year by year. Due to rapid development and the high degree of malignancy associated with HCC, early diagnosis is difficult and the prognosis is poor. Traditional radiotherapy and chemotherapy do not significantly improve the survival rates of patients. Despite advances in

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immunotherapy and the development of targeted therapies for HCC, the high invasiveness and therapeutic resistance of HCC tumors often lead to poor prognosis. The tumor initiation potential results from TICs within the tumor owns stem cell related properties, which has self-renewal and strong tumorigenic capacity, and heterogeneous cell subsets exhibit varying degrees of tumor initiation potential. Therefore, the identification of molecules related to liver cancer stem cells and the elucidation of their regulatory mechanisms are very important in controlling HCC recurrence and metastasis.

The pre-B-cell leukemia homeobox belongs to transcription factors classes which is the 3-amino acid loop extension (TALE) family and has conserved homology domains highly. The PBX subfamily consists of four members (PBX1 through PBX4) (Ramberg et al., 2016), and they usually bind to a specific DNA sequence. These interactions result the activation or inhibition transcription of target gene (Laurent et al., 2008). PBX3 is thought to be involved in tumor growth and development. It has been reported that PBX3 is expressed highly in various solid tumors, such as malignant prostate cancer (Ramberg et al., 2011), colorectal cancer, gastric cancer (Li et al., 2014), cervical cancer (Li et al., 2017), retroperitoneal leiomyoma (Panagopoulos et al., 2015), and hematological malignancies such as multiple myeloma (Yu et al., 2016). Acute myeloid leukemia (Qin et al., 2016), and acute monocytic leukemia (Dickson et al., 2013). Using two Hep-12, HCC, Hep-11 cells, which represented non-tumorigenic and TIC-enriched cell populations (Xu et al., 2010), our research group previously demonstrated that the VGA channel subunits like $\alpha 2\delta 1$ can be used as a marker for hepatoma CSCs (Sainz and Heesch, 2013; Zhao et al., 2013; Han et al., 2015).

It was also demonstrated that Huh7 $\alpha 2\delta 1^+$ cells have stem cell characteristics. $\alpha 2\delta 1^+$ cells are able to form more spheroids, more tumor formation, as compared with $\alpha 2\delta 1^-$ counterparts (Han et al., 2015). Subsequently, it was identified that PBX3 was an important molecule that is capable of regulating the stemness of hepatoma cells. Studies have shown that PBX3 was essential in the recombination of $\alpha 2\delta 1^-$ HCC cells into TICs with stem cell-like properties and is necessary for the maintenance of TIC properties in $\alpha 2\delta 1^+$ HCC cells (Han et al., 2015). Therefore, PBX3 can be guessed to be a potential therapy target for HCC and other tumors.

The application of PBX3 in the treatment of HCC requires an in-depth and detailed analysis of the regulatory mechanisms of PBX3 in hepatoma cells, and currently only a few studies have focused on PBX3 in hepatoma cells. In the current study, we conducted an investigation of the regulatory mechanisms of PBX3 in HCC. To confirm if PBX3 was post-translationally controlled by the UPS in hepatoma cells. Above of all, we blocked protein synthesis using cycloheximide, then pulse-chased PBX3 protein in Huh7 cells (Zheng et al., 2014). We found PBX3 protein was rapidly degraded and became undetectable within 4 h of CHX treatment, suggesting that the PBX3 protein has a short half-life and is extremely unstable. However, when we treated the hepatoma cells with the proteasome inhibitor MG132 while simultaneously monitoring PBX3 expression, we found that the protein levels decreased significantly, confirming that PBX3 is not degraded by the ubiquitin-proteasome system (UPS). This led us to hypothesize that there are additional mechanisms by which MG132 downregulates the expression of the PBX3 protein in hepatoma cells. In our previous research, we performed a genome-wide analysis of miRNA expression of the Hep-12&Hep-11 cell lines through miRNA chips, and then used soft agar clone formation experiments for screening and related functional experiments to verify. Hep-12, which is rich in tumor stem cells, has low expression and can inhibit hep-12 stem miRNAs: let7c, miR200b, miR222, and miR424. Subsequently, the miRWalk algorithm was used to forecast the target genes of

four miRNAs, and functional experiments confirmed that PBX3 was one of their targets.

It has been reported that Argonaute2 protein, which was an important component of RNA-induced silencing complex (RISC), and it played important role to miRNA generation (Zhang et al., 2018). In this study, we will explore how a proteasome inhibitor down-regulate PBX3 expression.

2. Material and methods

2.1. Cell lines

The Huh7 cell line was provided by the Japan Society for the Promotion of Science (Tokyo, Japan). The Hep-12&Hep-11 HCC cell lines were isolated from liver cancer tissues respectively. Hep12 cells were characterized by different stem cell markers such as Sox2, Nanog, Oct4. More tumor formations and more spheroids were examined in the Hep12 cells (Xu et al., 2010). Huh7, Hep-11, and Hep-12 cells were cultured in RPMI 1640 medium, and 293FT cells were cultured in DMEM. These media were supplemented with 10% fetal bovine serum (FBS), 100 mg mL⁻¹ streptomycin and 100 U mL⁻¹ penicillin. All experimental cells were cultured in 5% CO₂, 37 °C. Before initiation of the study, the cells were identified using polymorphic short tandem repeat site to ensure they were not contaminated with mycoplasma.

2.2. CHX, MG132, and bortezomib drug treatment

Planting 5×10^5 Huh7 and Hep12 cells into 6-well plate, then treated Huh7 cells with CHX (Boston, USA) for 0, 0.5, 1, 2, 4 h, or bortezomib (selleck, Houston, Texas, USA) for 0, 2, 4 h, and treated Huh7 and Hep12 cells with MG132 (selleck, Houston, Texas, USA) for 0,2,4 h.

2.3. Protein extraction and western blot analysis

Proteins were extracted on ice using RIPA buffer (Solarbio Science & Technology Co., Ltd, Beijing, China) that contained all proteases and a phosphatase inhibitor cocktail. The extracted proteins were separated using 10% SDS-PAGE, blotted onto PVDF membranes, and probed using rabbit anti-PBX3 (1:3000, Abcam, Cambridge, UK, #Ab109173), rabbit anti-Argonaute2 (1:2000, CST, #2897), rabbit anti-HA (1:2000 dilution, CST, #3724), and rabbit anti-GAPDH (1:5000, CST, #2118). Subsequently, the protein was incubated with horseradish-peroxidase-conjugated goat anti-rabbit (1:100,000, Santa Cruz Biotechnology) as the secondary antibody. Chemiluminescence detection experimental protein analysis results.

2.4. Total RNA extraction and qRT-PCR

Total RNA was extracted using Invitrogen kit. RNA Quantity was measured by 260&280 nm absorbance value. As to mRNA detection, cDNAs were generated from total RNA using Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Invitrogen). For mature miRNA quantification, 200 ng total RNA was polyadenylated using polyA polymerase, followed by reverse transcription with oligo-dT adapter primer (5'-GCCAGCACAGAATTAATACGACT CACTATAGTGTTCCTTTTTCVN-3'). The mRNA expression was detected by fluorescent RNA on ABI7500 PCR instrument, and the internal reference was GAPDH/UG for miRNA quantification. All primers used shows in Table 1. Fold change was calculated using the $2^{-\Delta\Delta Ct}$ method according to the equation: $\Delta Ct = Ct (\text{Target}) - Ct (\text{Reference})$.

Table 1
Primer sequences used for gene expression test.

Primer	Forward	Reverse
GAPDH	5'GACCCCTTCATTGACCTCAAC	5'CTTCTCCATGGTGGTGAAGA
U6	CGCTTCGGCAGCACATATAC	TTCACGAATTTGCGTGCAT
PBX3	5'CAAGTCGGAGCCAATGTG	5'ATGTAGCTCAGGGAAAAGTG
Argonaut2	5'CTAGCTAGCGCCACCATGTACTCGGGA	5'CGGGATCCAGCAAAGTACATGGTGCC
Let7c	5'GTTGAGGTAGTAGGTTGTATGGTT	5'GCGAGCACAGAATTAATACGAC
miR-200b	5'TAATACTGCCTGGTAATGATGAC	5'GCGAGCACAGAATTAATACGAC
miR-222	5'AGCTACATCTGGCTACTGGGT	5'GCGAGCACAGAATTAATACGAC
miR-424	5'GCAGCAGCAATTCATGTTTTGAA	5'GCGAGCACAGAATTAATACGAC
miR-122	5'TGGAGTGTGACAATGGTGTGTTG	5'GCGAGCACAGAATTAATACGAC
miR-126	5'TCGTACCGTGAGTAATAATGCG	5'GCGAGCACAGAATTAATACGAC
miR-130b	5'ACTCTTCCCTGTGCACTACT	5'GCGAGCACAGAATTAATACGAC

2.5. Lentiviral vector construction and transient transfection

All constructs were measured using standard DNA recombination techniques. Argonaute2 sequence was amplified from the Hep11 genomic DNA using PCR and cloned into the NheI and BamHI restriction sites of the lentiviral shuttle vector pELNS-DDK (Invitrogen) to generate the human pELNS-Argonaute2-DDK plasmid. Forward and reverse primers sequences used to Argonaute2 cloning were CTAGCTAGCGCCACCATGTACTCGGGA (NheI) and CGGGATCCAGCAAAGTACATGGTGCC (BamHI), respectively. The HA-Ub vector was constructed using the procedures detailed in our previous study. All of the constructs were verified by sequencing. To transiently overexpress Argonaute2, 293T cells were co-transfected with 6 µg of pELNS-Ago2-DDK and 6 µg of HA-Ub using polyethylenimine (Tiansheng et al., 2020) reagent (Poly-sciences) in a 10 cm dish for two days.

2.6. Statistical analyses

The data from three independent experiments were presented as mean ± SD (standard deviation). Data assayed using one-way non-parametric ANOVA followed by Student-Newman-Keuls post hoc test. $P < 0.05$ considered these data differences to be statistically significant.

3. Results

3.1. Proteasome inhibitors suppress the expression of the PBX3 in hepatoma cells

Compared PBX3 protein expression in Hep11 & Hep12 cell lines, which represented non-tumorigenic and TIC-enriched cell populations, respectively, to verify that PBX3 is associated with the stemness of hepatoma cells. Western blot analysis demonstrated that the protein levels of PBX3 were higher in Hep12 cells than in Hep11 cells (Fig. 1A), which was in accordance with previous results published by our laboratory (Han et al., 2015) and suggests PBX3 regulates the TIC properties of hepatocellular carcinoma (HCC) tumor cells. While PBX3 is a potential target in the treatment of HCC, the mechanism of its regulation is not yet fully understood.

To further clarify the regulatory mechanism of PBX3 in cancer cells, we used cells of the Huh7 cell line that are commonly used in laboratories because they have high malignancy and exhibit high levels of PBX3 expression. Cycloheximide (CHX) was used to block the protein synthesis process in cells, and the expression of PBX3 protein in Huh7 cells was tracked by pulse to detect its stability. The PBX3 protein was rapidly degraded and became almost undetectable within 4 h of CHX treatment (Fig. 1B), suggesting that the PBX3 protein has a short half-life and is extremely unstable. It is believed that unstable proteins are susceptible to degradation by

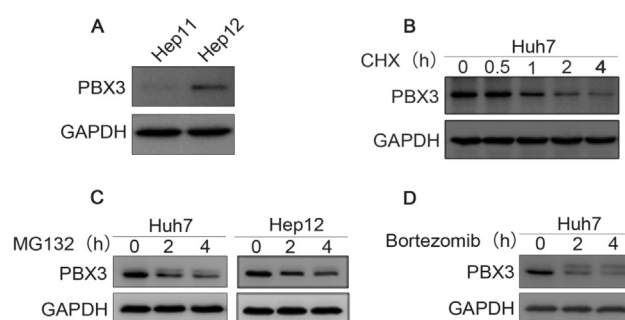


Fig. 1. Proteasome inhibitors suppress the protein expression of PBX3 in hepatoma cells.

the ubiquitin-proteasome system. However, we found that the PBX3 protein was down-regulated (Fig. 1C left panel), rather than upregulated, when Huh7 cells were treated with the proteasomal inhibitor MG132. Furthermore, when we used the same method to treat the hepatoma Hep12 cells, we obtained similar results (Fig. 1C right panel). We treated Huh7 cells with another proteasome inhibitor, bortezomib, and found that the PBX3 protein remained inhibited (Fig. 1D). These data demonstrate that proteasome inhibitors can inhibit PBX3 protein expressions in hepatoma cells.

3.2. MG132 suppresses the expression of PBX3 mRNA in hepatoma cells

MG132 directly inhibits the expression of the PBX3 protein, indicating that PBX3 is not degraded via the ubiquitin-proteasome pathway. We hypothesized that MG132 regulates PBX3 by inhibiting the expression of PBX3 at the level of gene expression. To test our hypothesis, we treated Huh7 and Hep12 cells with MG132 for 24 h and measured the expression of PBX3. qPCR and western blot results demonstrate that the level of PBX3 mRNA (Fig. 2A,C) ($P < 0.05$) showed the same downward trend as the level of the PBX3 protein (Fig. 2B,D) after treatment of Huh7 cells with MG132. These data demonstrate that MG132 inhibits the expression of PBX3 at the level of gene expression.

3.3. MG132 inhibits the expression of PBX3

MicroRNAs (miRNAs) are a large family of short noncoding RNAs ranging from 20 to 25 nucleotides in length. Mature miRNAs are produced from long primary transcripts after a series of nuclease shear processings and assembled into RNA induced silencing complexes (RISC). mRNA stability or transcription and translation are negatively regulated by pairing with the 3'-untranslated region (UTRs) of the target gene and were functionally similar to stem cells (Wang et al., 2007; Marson et al., 2008; Tay et al., 2008;

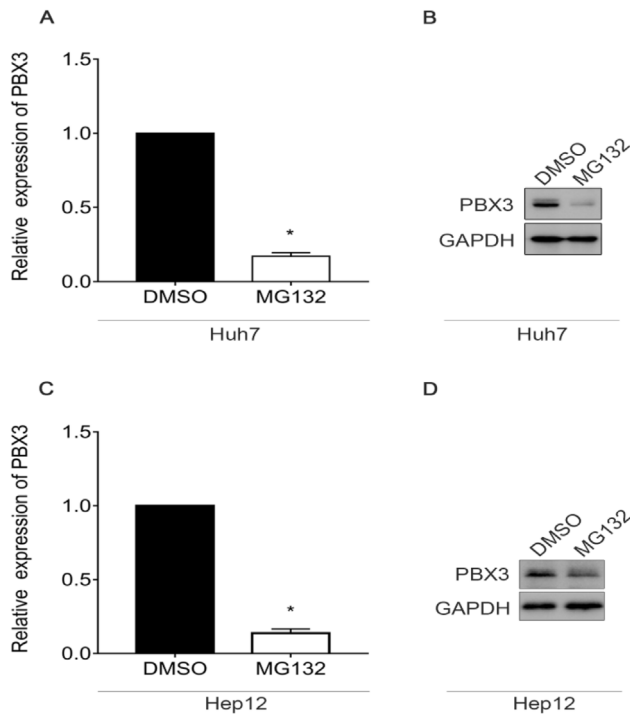


Fig. 2. MG132 inhibits the mRNA expression of PBX3 in hepatoma cells.

Ivey and Srivastava, 2010) and TICs or CSCs (Liu and Tang, 2011). In our previous studies, in previous experiments, we used trimer-enriched hep-12 and non-tumorigenic hep-11 cell lines, and performed genome-wide spectrum analysis and functional screening of miRNA expression using miRNA microarray analysis and soft AGAR method. Results showed mir-222, let-7c, mir-424, mir-200 could inhibit the dryness of hep-12 cells. At this time we verified that PBX3 was the common target for these four miRNAs (Han et al., 2015).

We next evaluated whether the regulation of PBX3 by MG132 is related to these miRNAs. We first measured the expression of these four miRNAs in Hep-11 and Hep-12 cells by RT-qPCR. The data showed that the expression levels of the four miRNAs were higher in the nontumorigenic Hep-11 cell line than in TIC-enriched Hep12 cells (Fig. 3A) ($P < 0.05$), indicating that the expression of these miRNAs was negatively correlated with the expression of PBX3. These results demonstrate that these four miRNAs can regulate the stemness of hepatoma cells and are consistent with results previously published by our laboratory (Han et al., 2015).

To determine whether the four miRNAs are involved in the inhibitory effect of MG132 on the expression of PBX3 in hepatoma cells, we treated Huh7 cells with 5 μ M MG132 for 24 h (Pandit and Gartel, 2011), intracellular PBX3 expression decreased with increasing dose (0,2,4,6,8 μ M) of MG132 treatment. However, when the dose of MG132 is too large (>5 μ M), the cell damage is obvious, so we used 5 μ M. And then we measured the expression of these four miRNAs using RT-qPCR. The data showed that the expression levels of miR-200b, let-7c, miR-424, miR-222 in the MG132 treatment group were upregulated nearly 2-fold compared to control group (Fig. 3B) ($P < 0.05$).

Furthermore, to determine whether MG132 is specific for the miRNAs studied, we measured the expression of random miRNAs (miR122, miR126, and miR130b; known to be unrelated to the stemness of HCC) after Huh7 cells were treated with MG132 for 24 h. We found that none of the miRNAs, except miR126, differed significantly from the control group (Fig. 3C), suggesting that the proteasome inhibitor MG132 has specificity for four miRNAs. To

further characterize this influence on MG132 on these miRNAs in hepatoma cells, the levels of expression of 4 miRNAs & PBX3 in Huh7 cells treated with 5 μ M MG132 for 0, 3, 6, 12, and 24 h were measured using RT-qPCR. The data showed that MG132 treatment increased the expression of the four miRNAs (Fig. 3D,E,F,G) ($P < 0.05$) and decreased the expression of PBX3 (Fig. 3H) ($P < 0.05$) in a time-dependent manner. A significant negative correlation was observed (Fig. 3I) between the designated miRNAs and PBX3 mRNA expression after the Huh7 cells were continuously subjected to MG132. Collectively, these data demonstrate that MG132 may up-regulate the expression of four miRNAs in Huh7 cells even this upregulation consequently results in the inhibition of PBX3 expression.

3.4. MG132 stabilizes the Argonaute2 protein by inhibiting its degradation by ubiquitination

The peptide-aldehyde proteasome inhibitor MG132 (carbobenzoxyl-L-leucyl-L-leucyl-L-leucinal) is a natural triterpene proteasome inhibitor derived from a Chinese medicinal plant and can inhibit 20S proteasome activity by covalently binding to the active site of the beta subunits; thus, MG132 effectively blocks the proteolytic activity of the 26S proteasome complex.

We next investigated the mechanism of MG132 regulation of miRNAs and whether MG132 affects the expression of some important proteins that induce miRNA expression, thereby indirectly affecting miRNA expression. It was reported that the Argonaute2 (Ago2) protein, which was an important component of the RNA-induced silencing complex (RISC), played an important role in the production of miRNAs (Zhang et al., 2009). We hypothesized that MG132 regulates miRNA levels by targeting the Ago2 protein.

Cyclohexanamine (CHX) was used to block protein synthesis, and then Ago2 protein expression was pulse tracked in Huh7 cells, so as to prove that Ago2 protein was regulated by ubiquitin proteome system (UPS) after translation in liver cancer cells. Ago2 protein was rapidly degraded upon CHX treatment (Fig. 4A). Subsequently, we treated Huh7 cells with MG132 and found that the treatment significantly increased Ago2 protein levels (Fig. 4B), confirming that the Ago2 protein is degraded by the UPS. In addition, we constructed a pELNS-Ago2-DDK plasmid and co-transfected it with HA-Ub into 293T cells. Two days after transfection, the cells were treated with 5 μ M MG132 for 12 h. The ubiquitinated Ago2 protein was detected using co-immunoprecipitation (Co-IP) and western blot assays. The results demonstrate that MG132 promotes the accumulation of ubiquitinated Ago2 protein (Fig. 4C). In summary, the data suggest the proteasome inhibitor MG132 suppresses the expression of PBX3 by targeting Ago2 and modulating the expression of four miRNAs.

4. Discussion

HCC is one of the most prevalent malignancies and the leading cause of cancer-related deaths worldwide (Wang et al., 2019). Local recurrence and distant metastasis often result in poor prognosis (Ringehan et al., 2017). As a member of the PBX family, PBX3 has been widely reported to be associated with tumor growth and progression. Studies have shown that PBX3 is highly expressed in a variety of solid tumors, such as colorectal cancer, gastric cancer (Li et al., 2014), cervical cancer (Li et al., 2017), retroperitoneal leiomyomas (Panagopoulos et al., 2015), and in hematological malignancies such as multiple myeloma (Yu et al., 2016), acute myeloid leukemia (Qin et al., 2016), and acute monocytic leukemia acute (Dickson et al., 2013). Moreover, the expression level of PBX3 is largely related to the grade or pathological

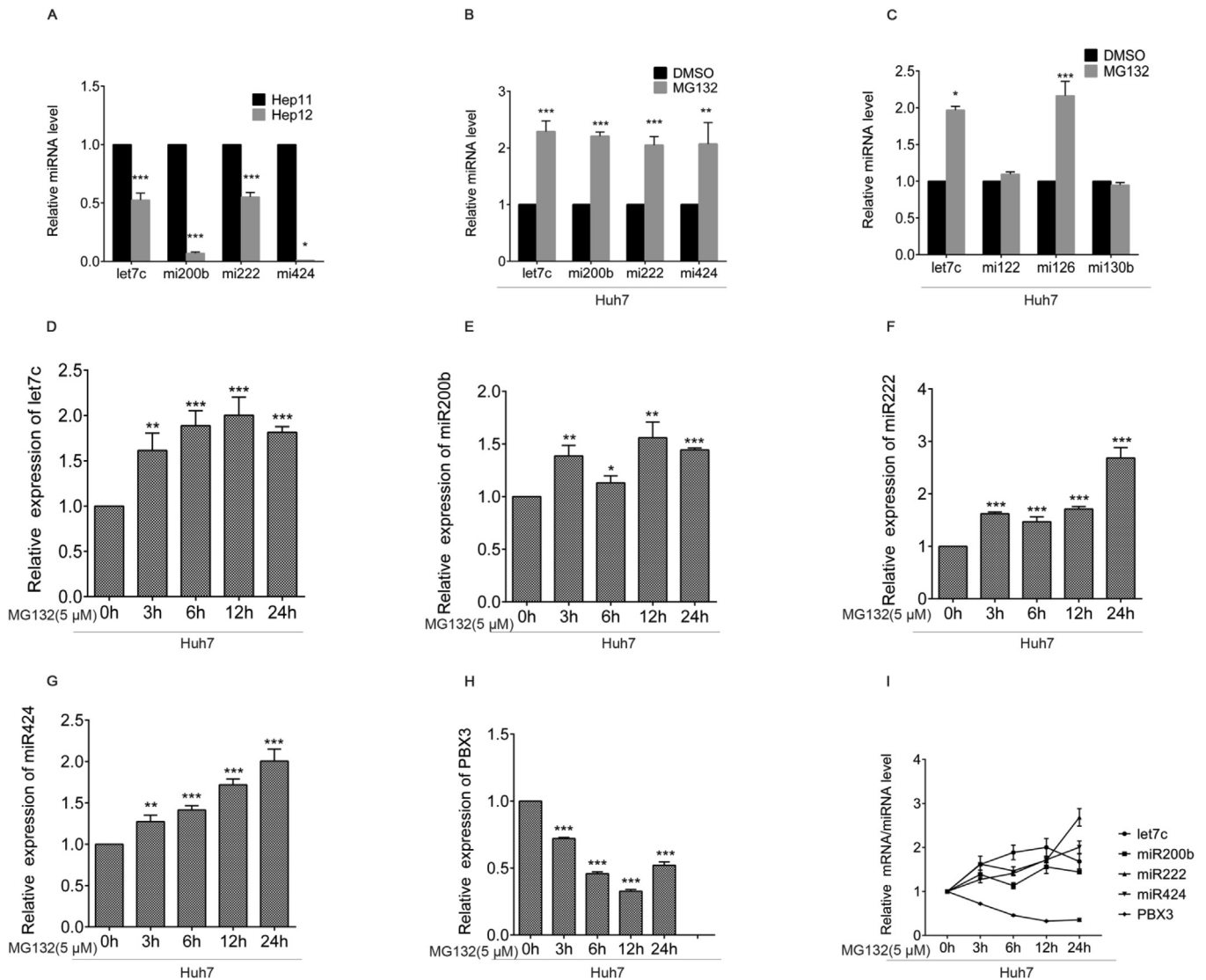


Fig. 3. MG132 inhibits the expression of PBX3 through up-regulation of four miRNAs.

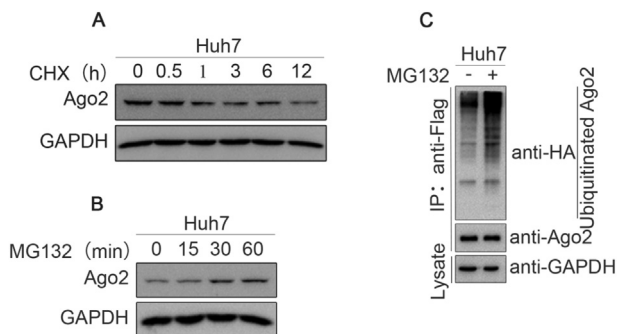


Fig. 4. MG132 stabilizes the Argonaute2 protein by inhibiting ubiquitination degradation.

stage of the tumor; the higher the degree of malignancy of the tumor, the higher the expression of PBX3 (Li et al., 2014, 2017).

Recently, PBX3 has been found to be closely related to the progression of HCC and is considered a potential biomarker in the prognosis and treatment of HCC (Han et al., 2016). PBX3 expression is sufficient for hepatoma cells to obtain the characteristics of

tumor-initiating cells and to maintain this property (Han et al., 2015). Therefore, PBX3 is a potential therapeutic target for HCC and other tumors. Elucidating the mechanism of regulation of the expression of PBX3 is particularly important for controlling the occurrence and development of HCC and some other tumors; however, only limited studies have been conducted with this focus. Therefore, we conducted a preliminary investigation of the regulatory mechanism of PBX3.

We confirmed the correlation between PBX3 and the stemness of HCC using western blotting, and we found that PBX3 had a short half-life after hepatoma cells were treated with CHX. However, when the two hepatocellular carcinoma cell lines Huh7 and Hep12 were treated with the proteasome inhibitor MG132, we found that the PBX3 protein was downregulated rather than up-regulated. A similar result was obtained after treatment of Huh7 cells with proteasome inhibitor bortezomib, suggesting the PBX3 protein was not degraded by the ubiquitin-proteasome pathway. Since proteasome inhibitors usually act to stabilize proteins that are unstable, inhibition of the PBX3 protein upon treatment of hepatoma cells with MG132 seems paradoxical.

To investigate the mechanism by which proteasome inhibitors regulate PBX3, we monitored the change in PBX3 at the gene level

after Huh7 cells were treated with MG132. We found that the gene expression changes were consistent with protein changes, indicating that MG132 inhibits the expression of PBX3 at the genetic level. Regulation of gene expression can be performed at multiple levels, including regulation at the gene level, transcription level, post-transcription level, translation level, and post-translational level. Whether MG132 directly affects PBX3 expression through promoter regulation does require further research and exclusion. We will explore it in subsequent experiments.

The miRNA pathway is also an important regulatory pathway. miRNAs silence gene expression at the post-transcriptional level to alter the cell phenotype by complementary pairing with target mRNAs. Previous studies have found that four miRNAs can regulate PBX3 and affect the stemness of hepatoma cells. We hypothesized that the proteasome inhibitor MG132 affects the expression of these miRNAs. We measured the levels of specific miRNAs after Huh7 cells were treated with MG132 and found that MG132 upregulated the expression of four miRNAs in a time-dependent manner. Moreover, the expression of PBX3 was negatively correlated with miRNA expression during this process.

We tested several elements such as Argonaute, exportin 5 and drosha which regulate miRNA, we found that proteasome inhibitors had no effect on exportin 5 and drosha. As an isoform of Argonaute, Ago2 is a common miRNA processing factor that can potentially elevate the levels of certain miRNAs. It has been reported that stable, long-term overexpression of Ago2 induces the production of a number of miRNAs, such as the let-7 family of miRNAs in 293T cells (Zhang et al., 2009).

Moreover, studies have shown that the Ago2 protein can be degraded by the ubiquitin-proteasome pathway. Therefore, we hypothesized that MG132 upregulates relevant miRNAs by stabilizing the expression of Ago2, ultimately leading to inhibition of PBX3 expression. In the present study, we demonstrated that the increase in Ago2 was accompanied by an increase in miRNAs after Huh7 cells were exposed to MG132. In addition, we mainly wanted to explore whether Argonaute2 could be degraded by ubiquitination, an inherent property of proteins that would not be altered by treatment with either 293T cells or Huh 7 cells. 293T cells were used because they were easier to transfect and easier to observe using. Using co-transfection, co-immunoprecipitation (Co-IP), and western blot assays, we also demonstrated that MG132 stabilizes its expression by inhibiting the degradation of the Ago2 protein via the ubiquitin-proteasome pathway.

Collectively, our results demonstrate that proteasome inhibitor MG132 inhibits the degradation of Ago2 via the ubiquitin-proteasome pathway and promotes the accumulation of Ago2. Thus, MG132 can stabilize the expression of the miRNA processing factor Ago2, enhancing the expression of four miRNAs, which results in the inhibition of PBX3 expression (Fig. 5). This affects the stemness of hepatoma cells.

5. Conclusion

In conclusion, our results confirmed that the PBX3 protein, which is closely related to HCC stemness, has a short half-life and is not degraded by the ubiquitin-proteasome pathway. In contrast to this result, we demonstrated that the proteasome inhibitor MG132 can affect the expression of four miRNAs by altering the stability of the Ago2 protein that is associated with their production, thereby indirectly affecting PBX3 expression and thus regulating the stemness of hepatoma cells. Our research further elucidates the mechanism of miRNA regulation of PBX3 and test another proteasome inhibitor, Lactacystinon, whether the proteasome inhibitor can reduce the stemness of HCC in vivo and in vitro. Further studies are needed to confirm this regulatory mechanism and to

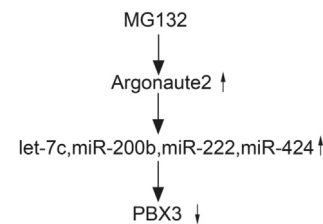


Fig. 5. Proposed model for the function of MG132 in regulating the Argonaute2-miRNA-PBX3 axis.

clarify how the Ago2 protein regulates miRNAs in hepatoma cells. The reason why PBX3, a short half-life protein, is not degraded by the UPS also warrants further investigation.

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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