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Original Article

HIF-1α suppresses myeloma progression by targeting Mcl-1

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Abstract: HIF-1α is involved in the carcinogenesis and progression of multiple types of cancer. However, the precise role of HIF-1α is unclear in multiple myeloma. Through the qRT-PCR and CCK-8 assays, we demonstrated that silencing the expression of HIF-1α and Mcl-1, MM proliferation can be decreased and apoptosis can be induced. Next, using the GEO database, we found that Mcl-1 was increased in MMs. Mcl-1 overexpression counterbalanced the tumor suppressing effect of siHIF-1α on MM apoptosis. Additionally, HIF-1α acting as a transcription factor, could directly target the promoter region of Mcl-1 to promote Mcl-1 expression. Based on the experimental result, our findings strongly suggest that HIF-1α regulated the progression of MMs by directly targeting the Mcl-1.

Keywords: HIF-1α, Mcl-1, MMs, proliferation, apoptosis

Introduction

Multiple myeloma (MM), characterized by uncontrolled proliferation of plasma cells, is a plasma cell malignancy [1]. MM accounts for 1% of all cancers and represents 10% of all hematologic malignancies [2], and is the second most common hematologic malignancy. Recently, although the survival rate of MM patients has increased because of rapid development in chemotherapy and novel autologous hematopoietic stem cell transplantation, the uncontrolled tumor metastasis and acquired drug resistance are the main reason for low short-term survival rates [3]. Therefore, it is a pressing requirement to identify the potential molecular mechanisms of MM proliferation, which remains vital for the development of novel therapeutic strategies.

Hypoxia-inducible factor 1 (HIF-1), as a significant heterodimeric transcription factor, contains two subunits. One is an oxygen-labile α subunit and another is a constitutive β subunit. Under hypoxia, HIF-1α stabilizes and rapid0 ly accumulates in cells, leading to hypoxia-induced responses [4]. As an important regulator of oxygen balance, growing evidence indicates that HIF-1α is intricately involved in a large number of biologic processes, including cell proliferation, apoptosis, and migration and invasion [5]. Downregulation of HIF-1α in gastric cancer inhibits SGC7901 cell proliferation and induces apoptosis [6]. In the research on gastric cancer, HIF-1α can go through the PI3K/AKT pathway to regulate the migration and invasion of gastric cancer cell lines [7]. In the MM, HIF-1α also plays oncogenic roles in tumor multidrug resistance and poor prognosis [8]. However, there is little research on the relationship between HIF-1α and Mcl-1 in MM.

The myeloid cell leukemia-1 (Mcl-1) is a member of the Bcl-2 gene family. The family proteins of Bcl-2 act as crucial regulators of cell survival and death [9]. Antiapoptotic Mcl-1 participated in intracellular mechanisms of apoptotic and validated anti-cancer targets [10]. A large number of examples of overexpression of Mcl-1 have been found in many cancers, such as breast cancer [11, 12], ovarian cancer [13], lung cancer [14], and renal cancer [15]. Mcl-1

expression is regulated by different mechanisms, such as transcriptional, post-transcriptional, and so on. However, it remains a great challenge to know the function of HIF-1α in Mcl-1 expression, especially in MM.

Herein, we reveal that HIF-1α mediating Mcl-1 plays a key role in the regulation of progression of MMs based on gain-and loss-of-function of HIF-1α and Mcl-1. This is the first time that the correlation between HIF-1α and Mcl-1 has been directly illustrated. Thus, HIF-1α and Mcl-1 may serve as potential gene therapy in MM.

Materials and methods

Cell lines

PRMI8226 cells were cultured in 1640 medium, supplemented with 10% fetal bovine se0 rum (FBS) at 37°C in 5% CO_2.

Plasmid and siRNA

The small interfering RNA (siRNA) targeting HIF-1α and Mcl-1 were purchased from the company (TranSheep Bio-Tech Co, Ltd, Shanghai, China). The plasmid of HIF-1α and Mcl-1 were purchased from the company (Hanbio, Shanghai, China).

Real-time PCR

Total RNA was isolated from the cells using RNAiso Plus reagent (Takara) according to the manufacturer's protocol. qRT-PCR was performed according to the methods described previously [16]. The sequences of PCR primers were as follows: forward, 5'-GGAGGAGGAGGACGAGTTGTA-3', reverse, 5'-TTTGTTACGCCGTCGCTGA-3', for Mcl-1, and forward, 5'-CCTCACCAAACAGAGCAGGAA-3', and reverse, 5'-ATGATCGTCTGGCTGCTGTAAT-3', for HIF-1α; forward, 5'-ACGTGGACATCCGCAAAGACC-3', and reverse, 5'-CCTTCTGCATCCTGTCGGCAA-3', for β-actin.

Cell proliferation assay

Cell proliferation was analyzed using the CCK-8 assay. CCK-8 assay was performed according to the methods described previously [17].

Cell apoptosis analysis

Cell apoptosis was analyzed using an Annexin-V FITC/PI Apoptosis Detection Kit (KeyGEN BioTECH, Nanjing, China), according to the manufacturer's instructions. Cells were seeded into 12-well plates at a density of 1×10^6 cells per well in triplicate, and 48 h after transfection, they were analyzed using flow cytometry (FACSort, Becton). Apoptotic populations were determined using ModFit software.

Western blot

Cells were obtained by lysing the cells in RIPA buffer, supplemented with protease inhibitor (Invitrogen). The protein concentration was calculated with a quantitative analyzer (GeneQuant pro RNA/DNA). Protein was then separated with an 8-10% SDS-PAGE (Invitrogen) gel; transferred to a nitrocellulose membrane; and incubated with the HIF-1α, Mcl-1, Bcl-2, Bax and GAPDH antibodies (Cell Signaling Technology; diluted 1/500). The membrane was washed three times with TBST, and was incubated with a goat anti-rabbit antibody (Bioworld; diluted 1/5000). Relative protein expression was then normalized to GAPDH levels in each sample.

Chromatin immunoprecipitation assay (ChIP)

The binding of HIF-1α to the promoter of Mcl-1 was verified using ChIP analysis. ChIP was performed according to the methods described previously [18].

Statistical analysis

Data are presented as mean ± SEM of at least three independent experiments. Student's *t*-test was used to compare two groups. All differences were considered significant at $P < 0.05$.

Results

HIF-1α and Mcl-1 expression levels in vitro

To explore the function of HIF-1α and Mcl-1 in MM cells, the expression of HIF-1α and Mcl-1 were silenced in PRMI8226 cells by RNAi, siHIF-1α, and siMcl-1 respectively. The mRNA expression levels of HIF-1α and Mcl-1 could be specifically knocked-down by siRNA in PRMI-8226 cells (**Figure 1A** and **1B**). Furthermore, we constructed HIF-1α and Mcl-1 overexpression vectors and transfected them into PRMI8226 cells respectively. In the **Figure 1A**,

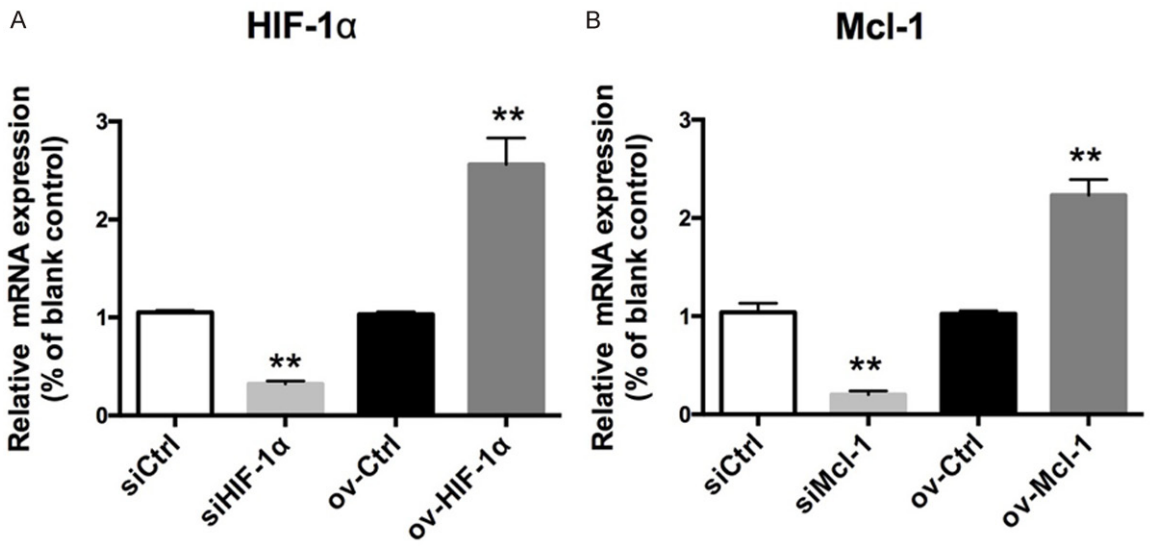

Figure 1. Expression of HIF-1α and Mcl-1 in MM cell lines. A. The expression levels of HIF-1α and Mcl-1 were measured by qRT-PCR in PRMI8226 cells transfected with siHIF-1α, siMcl-1 or their controls, respectively. B. MMs were transfected with HIF-1α vector, Mcl-1 vector, or control vectors (*: $P < 0.05$, **: $P < 0.01$).

expression of HIF-1α showed a more than 2-fold increase in PRMI8226 cells compared with controls, which is consistent with the result of Mcl-1 in the MM cells (**Figure 1B**), suggesting that siRNA (siHIF-1α and siMcl-1) and over-expression vector (HIF-1α and Mcl-1) worked in MMs.

HIF-1α and Mcl-1 expression levels affect PRMI8226 cells proliferation in vitro

To examine the proliferative role of HIF-1α and Mcl-1 in human MM cells, siHIF-1α and siMcl-1 had been transfected into PRMI8226 cells respectively. With the use of the CCK8 assay, silencing HIF-1α and Mcl-1 resulted in suppressed cell viability (**Figure 2A**). However, overexpression of HIF-1α and Mcl-1 could not affect the cell viability (**Figure 2B**). Restoration of Mcl-1 did not counteract the effects of HIF-1α in cell proliferation. These results indicate that HIF-1α and Mcl-1 may act as tumor oncogenes to regulate the progression of MMs.

HIF-1α and Mcl-1 induce apoptosis of MMs

To investigate the role of HIF-1α and Mcl-1 in PRMI8226 cells further, cell apoptosis analysis was applied. The silencing of HIF-1α and Mcl-1 resulted in the promotion of cell apoptosis. Over-expression of Mcl-1 rescued the the tumor suppressing effect of siHIF-1α on MM apoptosis (**Figure 3A**). Western blotting was used to identify the expression of apoptosis-related genes, Bcl2 and Bax. The results showed that down-expression of HIF-1α and Mcl-1 decreased the expression of Bcl-2, and increased Bax (**Figure 3B** and **3C**). These results demonstrated that HIF-1α and Mcl-1 played the key role of an oncogene in MM apoptosis.

HIF-1α activates Mcl-1 expression

To verify the relationship between HIF-1α and Mcl-1, PRMI8226 cells were divided into following treatment groups, blank, transfected with siHIF-1α, siMcl-1, co-transfected with siHIF-1α and ov-Mcl-1, ov-HIF-1α and siMcl-1, ov-HIF-1α and NC (negative control), ov-Mcl-1 and NC. Using qRT-PCR, we identified the HIF-1α expression in different treatment groups. The expression of HIF-1α did not change when the Mcl-1 increased. Meanwhile, co-transfection with siHIF-1α and ov-Mcl-1 in MM cells, similar to the effect of ov-Mcl-1 in PRMI8226 cells, Mcl-1 did not regulate the expression of HIF-1α (**Figure 4A**). To investigate the role of HIF-1α in Mcl-1 expression, the expression of Mcl-1 was measured in different transfected MM cells, which indicate that the expression of Mcl-1 decreased in MM cells with of silencing HIF-1α. Besides, the overexpression of Mcl-1 in transfected siHIF-1α cells could rescue the effects of HIF-1α on Mcl-1 expression levels

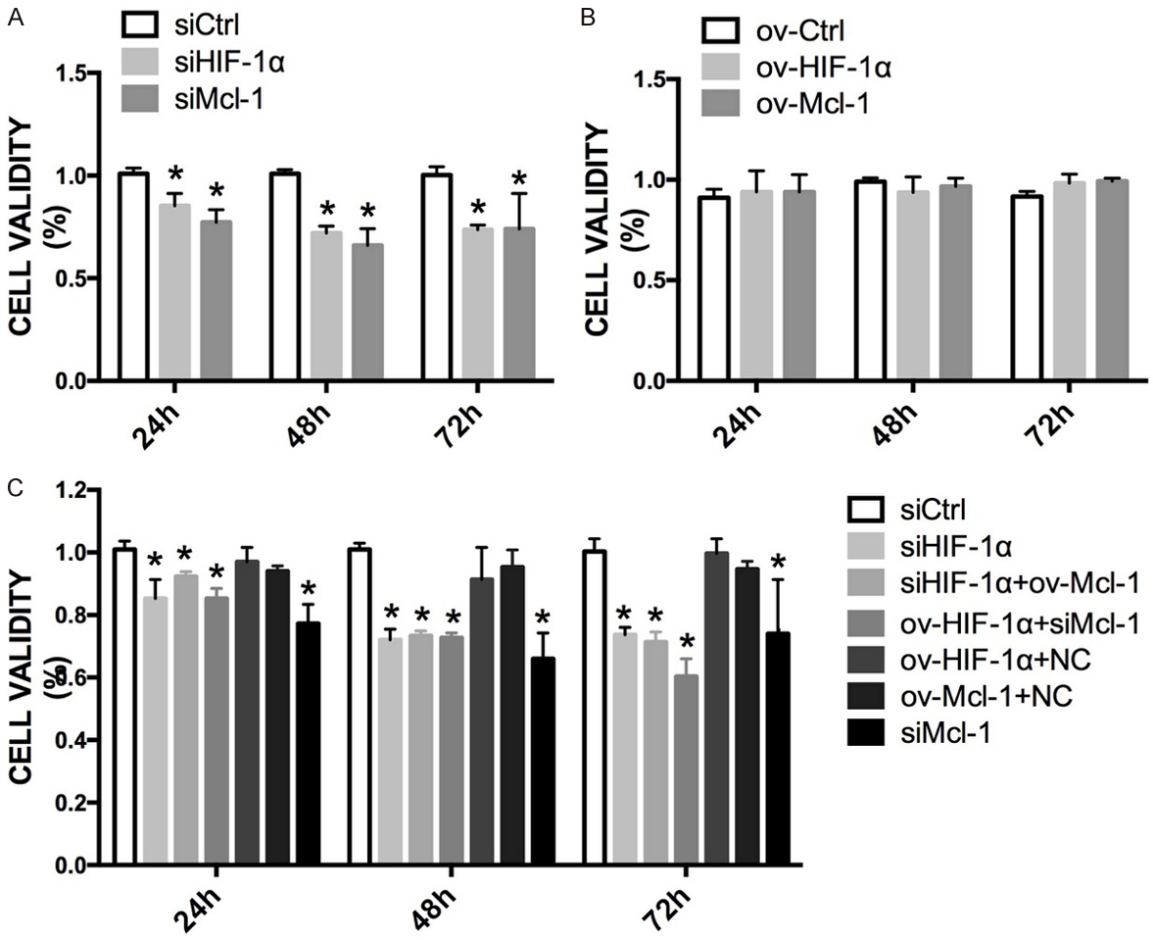

Figure 2. HIF-1α and Mcl-1 affect MM proliferation. A, B. CCK-8 assay was performed to test the growth of MMs treated with siHIF-1α, siMcl-1, HIF-1α, and Mcl-1 vector. C. CCK-8 assay was performed to test the growth of MMs treated with siCtrl, siHIF-1α, siHIF-1α+ov-Mcl-1, ov-HIF-1α+siMcl-1, ov-HIF-1α+NC, ov-Mcl-1+NC and siMcl-1 (*: $P < 0.05$, **: $P < 0.01$).

(**Figure 4B**). Furthermore, the expression of HIF-1α affected the expression of Mcl-1 at the protein level (**Figure 4C**). These data demonstrate that the expression of Mcl-1 is regulated by HIF-1α.

HIF-1α directly target Mcl-1

To elucidate the mechanism underlying the relationship HIF-1α and Mcl-1, the UCSC genome browser tool was used to identify putative binding sequences of HIF-1α on Mcl-1. There was one sequence, a promoter region of Mcl-1, that interacted with HIF-1α (**Figure 5A**). CHIP analysis was used to test whether HIF-1α directly targets Mcl-1 in MM cells. The CHIP result revealed HIF-1α with the putative binding site located in the Mcl-1 upstream region (**Figure 5B**). Taken together, all results suggest that HIF-1α might directly regulate Mcl-1 expression to affect the MM cell progression.

Discussion

Multiple myeloma is a blood system tumor, and its clinical manifestations are mainly bone destruction [19], which seriously threatens human health. The rapid growth of tumors can produce hypoxia in the microenvironment, which leads to the release of HIF-1α in the local environment, thereby increasing the secretion of other cytokines associated with the proliferation and angiogenesis of tumors. HIF-1α has become a key factor of tumorigenesis [20] and can be used as a marker of metastasis and recurrence of tumors [21]. A large number of studies have shown that down-regulation of HIF-1α could inhibit the migration of cancer

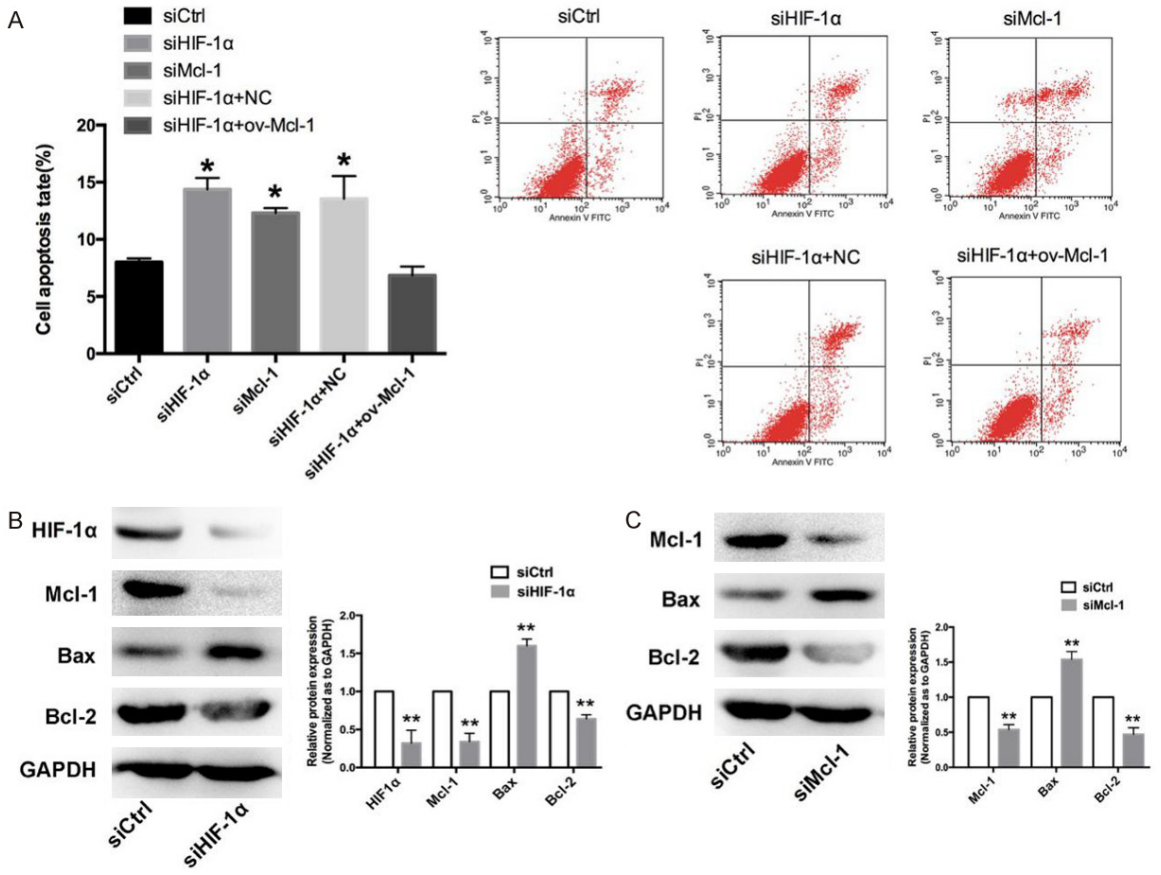

Figure 3. HIF-1α and Mcl-1 inhibit the apoptosis of MMs. A. Apoptosis was determined in MMs at 48 h after transfection with siCtrl, siHIF-1α, siMcl-1, siHIF-1α+NC and siHIF-1α+ov-Mcl-1, respectively. B. The expression of HIF-1α, Mcl-1, Bcl-xL and Bad were detected by western blotting. C. The expression of Mcl-1, Bcl-xL and Bad were detected by western blotting after siMcl-1 treatment (*: P < 0.05, **: P < 0.01).

cells [22], and HIF-1α has become an important target for cancer treatment [23, 24].

HIF-1α is involved in regulating the expression of BNip3 NIX, Noxa, and Mcl-1, thus promoting the anti-apoptotic effect of tumor cells. Mcl-1 could be highly expressed in a variety of malignant tumors, which is closely related to the degree of differentiation and prognosis of tumors [25, 26]. Mcl-1 is a type of anti-apoptotic protein, which blocks the suicide signaling pathway to maintain cell differentiation and maturation in many tissues [26]. More recent studies have exhibited that HIF-1α can regulate cancer cell progression via Mcl-1 [27]. However, the direct relationship between HIF-1α and Mcl-1 has not been clarified yet.

In this study, the GEO database was used to test the expression of Mcl-1in MMs. Mcl-1 was highly expressed in MMs (Figure S1). Furthermore, gain and loss of HIF-1α and Mcl-1 function was enforced, in order to observe the proliferation activity in MM cells. **Figure 2** showed that HIF-1α and Mcl-1, acting as oncogenes, participated in the regulation of MM progression. Using the siRNA to treat the MM cells, the proliferation of PRMI8226 cells was decreased. However, this effect had not been identified in the MM cells with high expression of HIF-1α and Mcl-1. We speculated this result could be induced by the expression levels of HIF-1α and Mcl-1 in MM cells. In **Figure 1**, only a 2-fold increase was observed in the HIF-1α and Mcl-1 transfected MM cells, so these expression levels of HIF-1α and Mcl-1 were not enough to trigger the cell signaling pathway-related with the progression of MMs.

Meanwhile, HIF-1α and Mcl-1 inhibited the apoptosis of MMs. Mcl-1 counterbalanced the

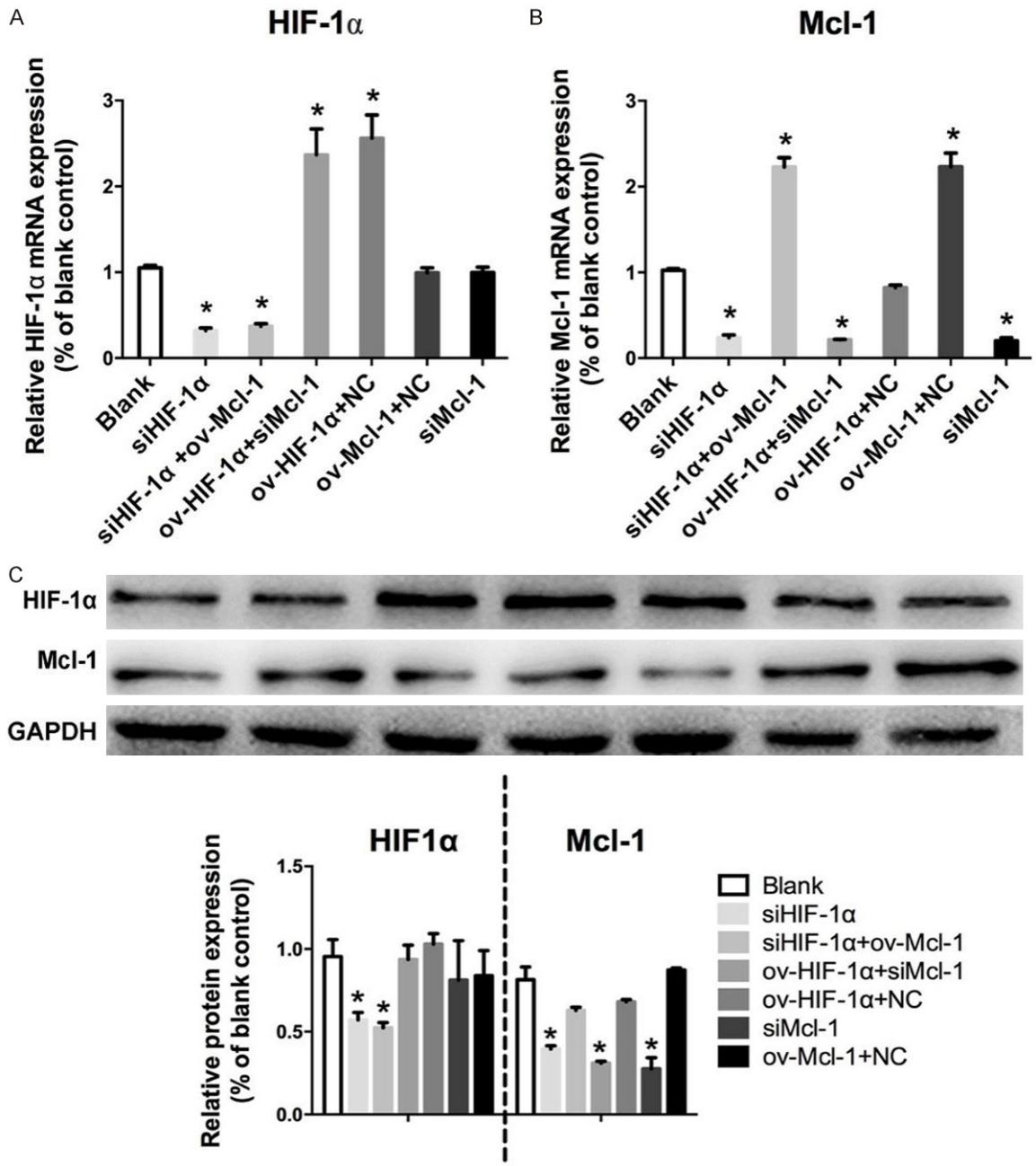

Figure 4. HIF-1α regulates the expression of Mcl-1 in MMs. A, B. mRNA expression levels of HIF-1α and Mcl-1 were measured by RT-PCR in different treatment groups of MMs. C. Protein expression of HIF-1α and Mcl-1 proteins in different treatment groups of MMs were analyzed by western blotting (*: $P < 0.05$, **: $P < 0.01$).

tumor suppressing effect of siHIF-1α on MM apoptosis (**Figure 3**).

In addition, to test the relationship between HIF-1α and Mcl-1, MMs were divided into different treatment groups. Silenced HIF-1α could decrease the expression of Mcl-1, and overexpression of Mcl-1 rescued the effect of HIF-1α on Mcl-1. While overexpression of HIF-1α did not work in regulating the expression of Mcl-1 at the mRNA and protein levels (**Figure 4**), maybe it also connected with the expression levels of HIF-1α in MMs.

MPrior work showed that HIF-1α as a transcription factor regulates gene expression. In gastric cancer, HIF-1α targeted the snail to affect epithelial-mesenchymal transition (EMT) of the CSCs [28]. HIF-1α-Ascl2-miR-200b regulatory feedback circuit modulated the EMT in colorec-

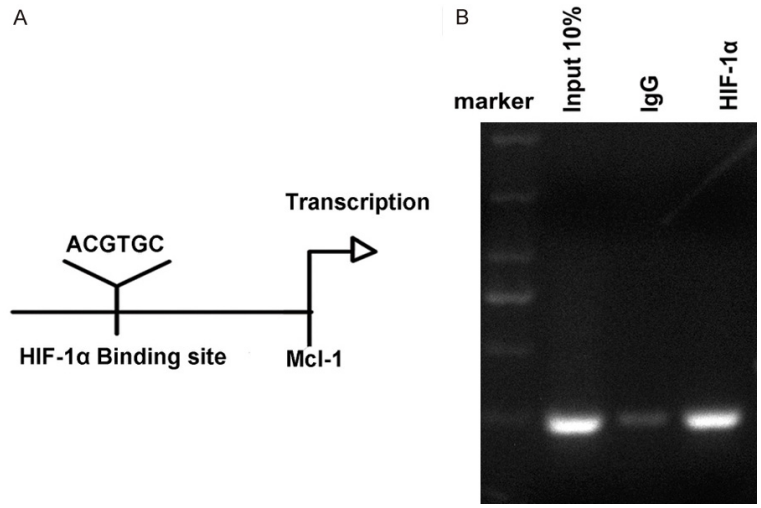

Figure 5. HIF-1α inhibits tumor progression by directly targeting Mcl-1. A. Schematic diagram of the Mcl-1 promoter region with one potential HIF-1α binding site. B. The interaction of HIF-1α with Mcl-1 was verified using ChIP assays (*: P < 0.05, **: P < 0.01).

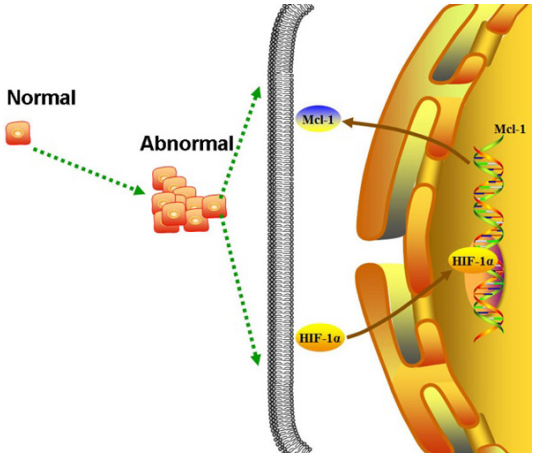

Figure 6. The proposed model for Mcl-1 was directly activated by HIF-1α. HIF-1α regulated the apoptosis of MMs by directly targeting the Mcl-1.

tal cancer cells [29]. Considering this fact, the bioinformatics tools (browser and jaspar) were used to predict the binding site of HIF-1α. We found that one sequence, at promoter region of the Mcl-1, interacted with HIF-1α. Meanwhile, we used a ChIP assay to verify the relationship between HIF-1α and Mcl-1, and showed that HIF-1α could directly target the promoter region of the Mcl-1 (**Figure 5**), suggesting that HIF-1α might participate in the regulation of Mcl-1expression in MM cells (**Figure 6**).

In this context, our result clarifies a deep understanding of the oncogenic role of HIF-1α and Mcl-1 during MM development. This is the first time that a relationship between HIF-1α and Mcl-1 was demonstrated in the MM cells.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

MM, Multiple myeloma; HIF-1, Hypoxia-inducible factor 1; VHL, von Hippel-Lindau; Mcl-1, The myeloid cell leukemia-1; siRNA, small interfering RNA; cDNA, complementary DNA.

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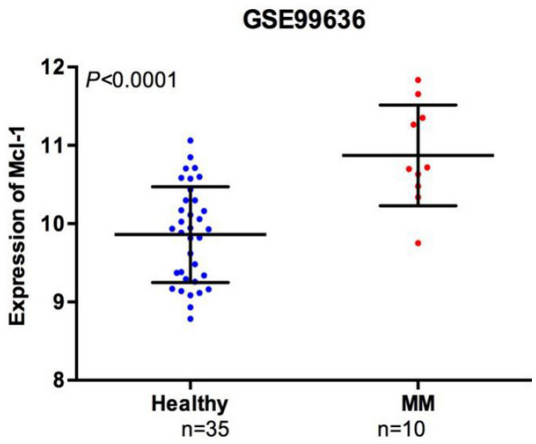

Figure S1. The expression of Mcl-1 in MMs. GEO database was used to test the expression of Mcl-1 in MMs.