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PSMB4 promotes multiple myeloma cell growth by activating NF-κB-miR-21 signaling

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ABSTRACT

Proteasomal subunit PSMB4, was recently identified as potential cancer driver genes in several tumors. However, the regulatory mechanism of PSMB4 on carcinogenesis process remains unclear. In this study, we investigated the expression and roles of PSMB4 in multiple myeloma (MM). We found a significant up-regulation of PSMB4 in MM plasma and cell lines. Ectopic overexpression of PSMB4 promoted cell growth and colony forming ability of MM cells, whereas inhibition of PSMB4 led to a decrease of such events. Furthermore, our results demonstrated the up-regulation of miR-21 and a positive correlation between the levels of miR-21 and PSMB4 in MM. Re-expression of miR-21 markedly rescued PSMB4 knockdown-mediated suppression of cell proliferation and clone-formation. Additionally, while enforced expression of PSMB4 profoundly increased NF-κB activity and the level of miR-21, PSMB4 knockdown or NF-κB inhibition suppressed miR-21 expression in MM cells. Taken together, our results demonstrated that PSMB4 regulated MM cell growth in part by activating NF-κB-miR-21 signaling, which may represent promising targets for novel specific therapies.

1. Introduction

Multiple myeloma (MM) is one of the most frequent hematological malignancies, characterized by the clonal proliferation of neoplastic plasma cells in the bone marrow [1]. However, the available treatment strategies for multiple myeloma, including autologous stem cell transplantation, chemotherapy, have not shown much benefit [2]. Although the use of proteasome inhibitors, such as bortezomib and carfilzomib, leads to significant improvements in response rates [3,4], identification of new actionable targets is also of great clinical need, which may offer new insights into targeted therapy against MM.

Proteasome is a giant protein complex responsible for protein degradation by proteolysis [5]. The breakdown of a large proportion of intracellular proteins is crucial to many important biological functions [6,7]. Emerging evidence indicates that, in various tumors, several proliferative and anti-apoptotic signaling pathways require proteasomal activity, suggesting the novel role of proteasome in tumorigenesis [8–10]. Indeed, proteasome inhibitors, bortezomib and carfilzomib, have proven effective against cancer cells in animal models and human trials by target the core proteolytic subunits PSMB5, PSMB6, and PSMB7 [11]. Now it is well established that the β4 subunit of the 20 S proteasome, PSMB4, regulates the assembly of the proteasome [12,13]. And targeting PSMB4 could potentially prevent the catalytic activity of all three proteolytic subunits [14]. Thus, PSMB4 may be the first proteasomal subunit shown to functions as an oncoprotein and serve as a novel potential therapeutic target for oncotherapy [14]. However, the role and regulatory mechanism of PSMB4 in the tumorigenesis of MM remain unclear.

Recent studies have indicated that microRNAs (miRNAs) can act as an oncogene to cause cancer, providing new targets for tumor treatments [15]. Emerging evidence indicates that miR-21 is an oncogenic miRNA in a wide variety of tumor types and is significantly increased in MM [16,17]. Overexpression of miR-21 is shown to promote tumor cell growth *in vitro* and in xenograft mouse models, reduce tumor cell apoptosis and increase cell invasiveness [18]. Substantial evidences showed that miR-21 plays a key role in the occurrence, progression, resistance and prognosis of MM

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[17,19]. Recent observations elucidated that the expression of miR-21 could be regulated by NF-κB by direct binding to its promoter [20]. The exact mechanisms in MM remain further investigation.

Here, we demonstrated that PSMB4 was significantly overexpressed in human MM plasma and cell lines. Through further functional investigations, we found that the up-regulation of PSMB4 increased, whereas down-regulation of PSMB4 decreased, the ability of cell proliferation and colony formation. Overexpression of PSMB4 was associated with increased NF-κB activity and miR-21 expression. PSMB4 knockdown or NF-κB inhibition-mediated suppression of cell growth were further rescued by re-expression of miR-21, suggesting the potential role of PSMB4- NF-κB-miR-21 axis in controlling MM cell growth.

2. Materials and methods

2.1. Tissue specimens, cell lines and transfection

The collection of tumor specimens from MM patients was approved by our Institutional Review Board (IRB). A panel of melanoma cell lines (IM9, OCI-MY5, SKMM2, RPMI8226, KMS12, OCI-MY1, OPM-2, MM1.R, MM1.S, U266, NCIH929 and LP1) was maintained per culture guidelines. pNF-κB-luc and control plasmids (Clontech, Mountain View, CA) were used to quantitatively examine NF-κB activity. Control pcDNA3.1 vector and pcDNA3.1-PSMB4, scrambled negative control RNA (pre-miR-control and anti-miR-control), pre-miR-21 and anti-miR-21 (Applied Biosystems, Foster City, CA, USA), control and PSMB4 specific siRNA were transfected into cells using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol.

2.2. Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from tissues and cell lines using TRIzol reagent (Invitrogen). Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Rockford, IL, USA) was used to obtain cDNA. The PSMB4 gene expression was validated using GoTaq qPCR Master Mix with SYBR green (Promega) with Actin as an internal control. To examine the expression of miRNAs, specific stem-loop RT primers (Applied Biosystems, Carlsbad, CA, USA) was used to obtain the cDNA, and then subjected to TaqMan microRNA assay (Applied Biosystems) using primers specific for miR-21 according to the manufacturer's protocol. U6 was used as endogenous control.

2.3. Western blot

After treatment, whole-cell lysates were prepared in RIPA buffer with protease inhibitors (Sigma, St. Louis, MI, USA), and subjected to Western blot using primary antibodies overnight, followed by a HRP-labeled second antibody. PSMB4 and IκBα antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-NF-κB p65 (pNF-κB, Ser536) were obtained from Abcam (Cambridge, MA, USA). Equal loading was verified using an anti-GAPDH antibody (Abcam).

2.4. Cell proliferation assay

Cell proliferation was detected by MTS assay. 3000 cells were seeded into 96-well plates after transfection. After being incubated for 4 days, cell proliferation was determined using CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

2.5. Soft-agar colony formation assay

Soft agar colony-forming experiment was used to detect the colony forming ability of MM cells. After transfection, IM9 and LP1 cells were harvested, pipetted well and mixed with pre-warmed agar. Then the mixture was added into 60-mm culture dish with soft agar-precoated. After 3 weeks, the colonies were stained with 0.04% crystal violet-2% ethanol in PBS. Colony was photographed under an inverted light microscope (Olympus, Tokyo, Japan).

2.6. Statistical analysis

All data were expressed as mean ± s.e.m. The significance was assessed by two tailed *t*-test or one-way ANOVA using the GraphPad Prism 5 (GraphPad Software, USA). The asterisk indicates a statistically significant difference: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3. Results

3.1. PSMB4 is overexpressed in MM plasma and cell lines

The expression of PSMB4 in plasmas of 21 multiple myeloma (MM) patients and 8 normal donors was examined by qRT-PCR. It was found that the plasma level of PSMB4 was significantly up-regulated in patients with MM compared with healthy control subjects ($P < 0.001$, Fig. 1A). Reanalysis of previously published dataset GSE39754 also confirmed the high expression of PSMB4 in MM ($P < 0.001$, Fig. 1B), indicating that PSMB4 may be contribute to the development of MM. In addition, the expression of PSMB4 in a panel of MM cell lines, was significantly increased compared with normal CD138 purified plasma cells (PCs) from healthy donors (Fig. 1C and D).

3.2. PSMB4 promotes MM cell growth and colony forming ability

To investigate the role that PSMB4 in the pathogenesis of MM, PSMB4-overexpressing or PSMB4 knockdown IM9 and LP1 MM cells were established (Fig. 2A). An increase in cell proliferation was found in IM9 and LP1 cells with PSMB4 overexpressed, whereas targeting PSMB4 with specific siRNA resulted in a significant decrease in cell growth (Fig. 2B). Furthermore, the effect of PSMB4 on anchorage-independent growth was determined by soft agar assays. As shown in Fig. 2C, the total number of colonies and average colony diameter were markedly increased by the transfection of PSMB4 vector ($P < 0.05$, compared to the control vector), and a decrease of such events was found in cells with PSMB4 knockdown. These findings suggested that PSMB4 may regulate cell growth and potential tumorigenicity of MM.

3.3. miR-21 is up-regulated by PSMB4 in MM plasma and cell lines

Previous studies have confirmed the role of miR-21 in the regulation of MM growth, survival and drug resistance, we further examined the expression of miR-21 in MM. It was found that miR-21 levels were up-regulated in PCs from MM patients as compared to that from normal donor (Fig. 3A). Strikingly, a strong positive correlation between miR-21 and PSMB4 expressions was found in MM ($r = 0.577$, $P < 0.01$), consistent with reanalysis of a previously published dataset GSE17306 ($r = 0.459$, $P < 0.001$, Fig. 3B). Furthermore, overexpression of PSMB4 significantly increased the expression of miR-21, whereas silencing of PSMB4 decreased miR-21 levels (Fig. 3C), suggesting the regulation of miR-21 by PSMB4. We also found that the up-regulation of PSMB4 increased, whereas

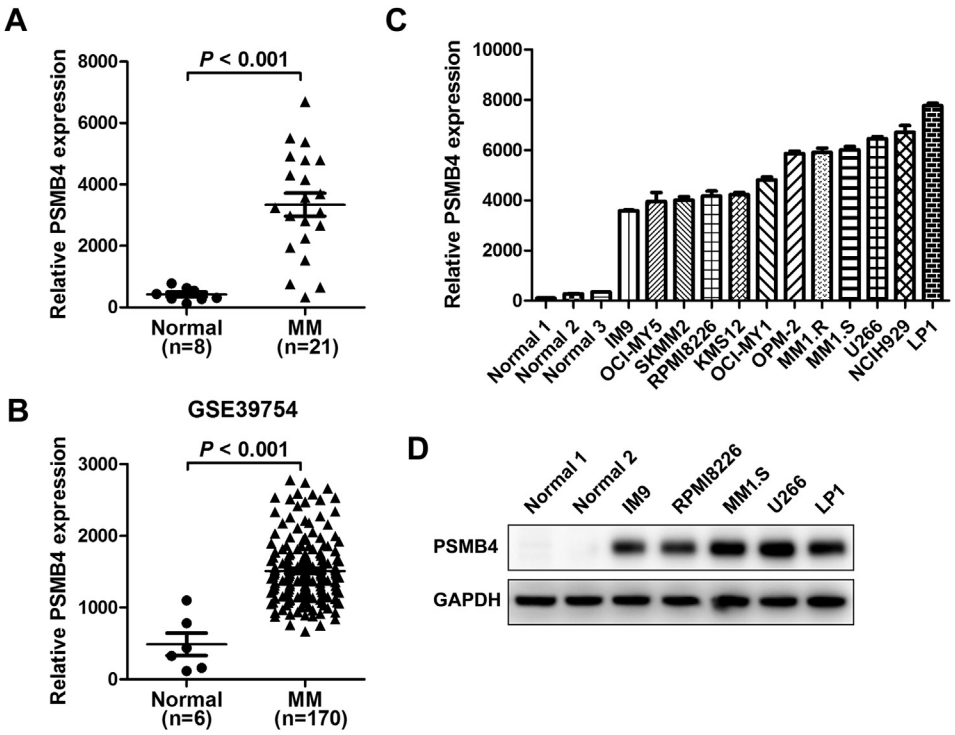

Fig. 1. PSMB4 is overexpressed in MM plasma and cell lines. (A) The expression of PSMB4 in 8 normal CD138 purified plasma cells (PCs) from healthy donors (Normal) and 21 multiple myeloma (MM) patients were examined by qRT-PCR. U6 snRNA was used as a loading control. (B) Reanalysis of PSMB4 expression in a previously published dataset GSE39754. The dataset was downloaded from the NCBI Gene Expression Omnibus (GEO) at http://www.ncbi.nlm.nih.gov. (C) qRT-PCR detecting the expression of PSMB4 in normal PCs and a panel of MM cell lines. (D) Western blot examining the PSMB4 expression in normal PCs and MM cell lines.

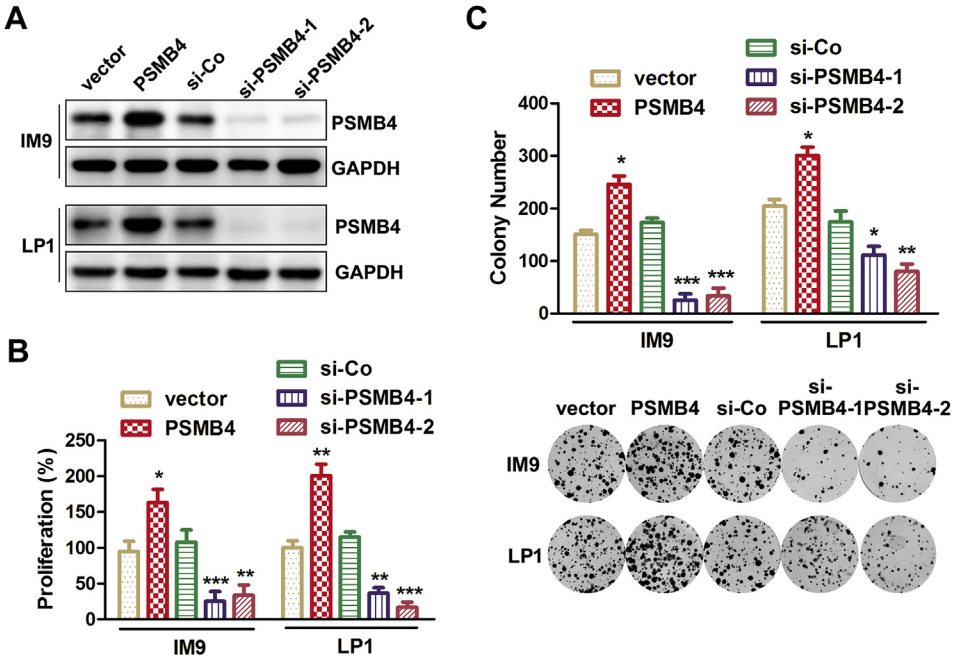

Fig. 2. PSMB4 promotes MM cell growth and colony forming ability. (A) IM9 and LP1 cells were transfected with control pcDNA3.1 or pcDNA3.1-PSMB4 vector, control or PSMB4-specific siRNA, and the expression of PSMB4 was detected by Western blot. (B) Cell proliferation was examined by MTS assay. (C) Cells were seeded into 6-well dishes, and the colony-formation ability was measured after transfection. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with corresponding control group.

down-regulation of PSMB4 decreased, the ability of cell proliferation and colony formation.

We also found an increased proliferation and colony number in cell with enforced miR-21 expression, and re-expression of miR-21 markedly rescued PSMB4 knockdown-mediated suppression of cell proliferation and colony forming ability of MM cells (Fig. 3D and E). Conversely, down-regulation of miR-21 by an inhibitor (anti-miR-21) markedly repressed the PSMB4-triggered proliferation and

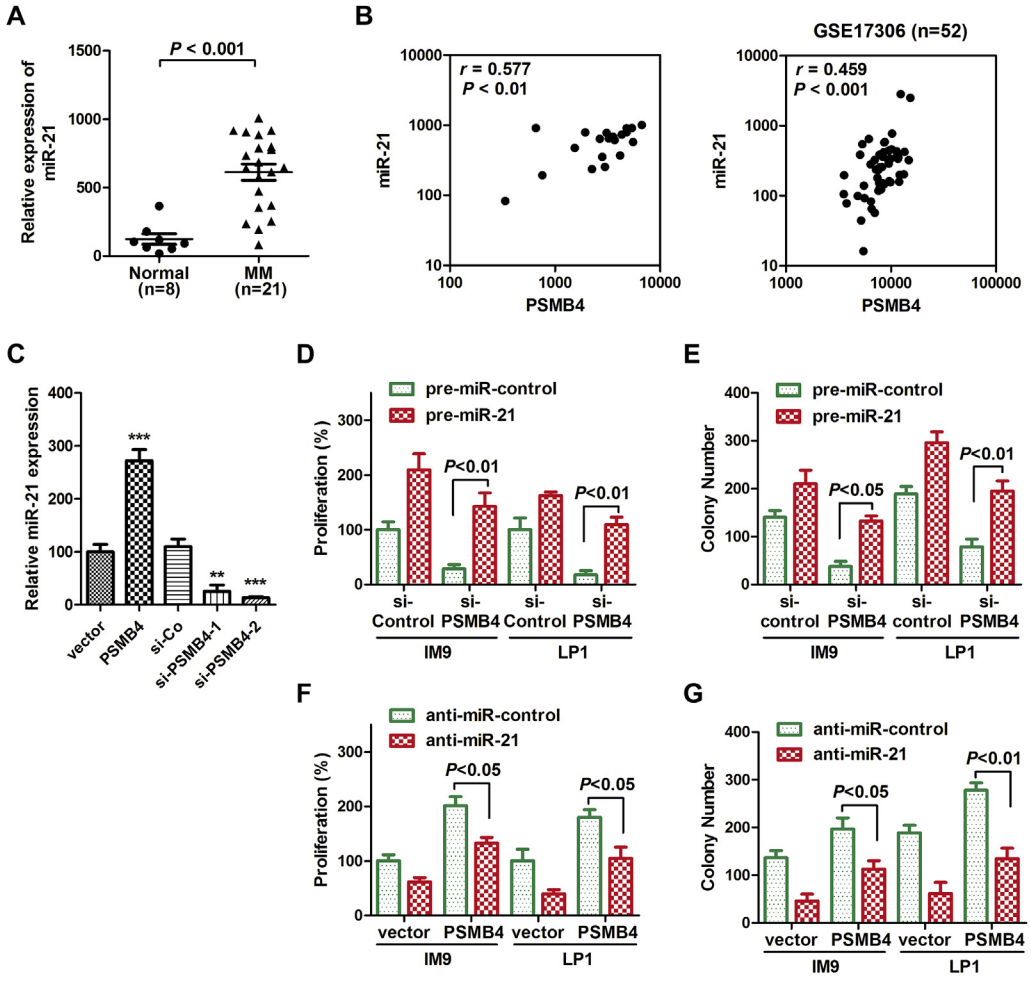

Fig. 3. miR-21 is up-regulated by PSMB4 in MM plasma and cell lines. (A) The expression of miR-21 was determined in normal PCs from healthy donors (Normal) and MM patients. (B) qRT-PCR evaluating the expression of miR-21 and PSMB4 in MM samples, and the correlation was calculated by GraphPad Prism program version 5. The correlation of miR-21 and PSMB4 levels in a previously published dataset was also analyzed. (C) The expression of miR-21 in PSMB4-overexpressing or PSMB4 knockdown IM9 and LP1 MM cells were examined by qRT-PCR. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with corresponding control group. IM9 and LP1 cells were transfected with si-PSMB4-1 and subsequently with pre-miR-21 expression vector, (D) the cell proliferation was determined by MTS assay, (E) and the colony forming ability was examined by soft agar assays. (F) The proliferation and (G) clone-formation were evaluated in PSMB4-overexpressing cells after transfection with control or anti-miR-21 miRNA inhibitors.

clone-formation (Fig. 3F and G). Thus, these results suggested that PSMB4 may promote MM cell growth, at least in part, by up-regulating miR-21expression.

3.4. PSMB4-induced miR-21 expression is modulated by NF-κB

Previous studies have confirmed that miR-21 could be directly regulated by the transcription factor NF-κB, and this process is crucial for NF-κB activation-mediated cell survival [21]. Our results from Western blot demonstrated a significant elevation in the phosphorylation of NF-κB p65 subunit in PSMB4-overexpressed cells, and a significant decrease in the PSMB4-silenced cells, compared to corresponding control cells (Fig. 4A). Similar results were found by determined the activity of NF-κB using luciferase reporter gene assay (Fig. 4B). Additionally, enforced PSMB4 expression led to a reduce in the level of IκBα, whereas silencing PSMB4 up-regulated IκBα expression, suggesting that PSMB4 may mediate NF-κB activation by inhibiting IκBα. To further validate that PSMB4-mediated miR-21 up-regulation occurs through NF-κB activation, we blocked the NF-κB pathway in PSMB4-overexpressing cells using an NF-κB inhibitor (BAY11-7082). As expected, while PSMB4 overexpression promoted NF-κB activation and miR-21 up-regulation, such effects were inhibited by BAY11-7082 (Fig. 4C). Additionally, BAY11-7082 also suppressed the stimulatory effect of PSMB4 overexpression on cell growth (Fig. 4D). Thus, these results indicated that the oncogenic function of PSMB4 may be dependent on activation of NF-κB-miR-21 signaling.

4. Discussion

Recently, PSMB4 has been identified as a potential driver oncogene by promoting both anchorage-independent growth and tumorigenesis [14,22]. It has been reported that PSMB4 is amplified and overexpressed in several primary and metastatic solid tumors, including hepatocellular carcinoma, ovarian cancer and gliomas [23–26]. However, the expression of PSMB4 in multiple myeloma (MM) and its functions underlying the malignant behavior remain unclear. In this study, we examined the expression of PSMB4 in plasmas of MM patients and normal donors, and found a significantly up-regulation of PSMB4 in MM, consistent with the results from re-analysis of a previously published dataset (Fig. 1), indicating that PSMB4 may play important roles in MM.

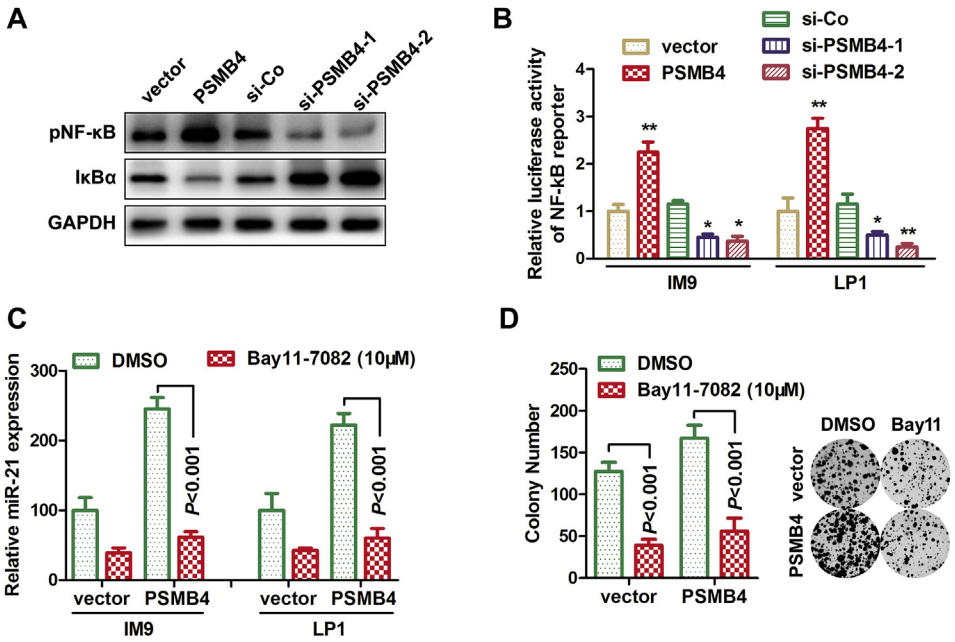

Fig. 4. PSMB4-induced miR-21 expression is modulated by NF-κB. (A) After transfection, the expression of pNF-κB and IκBα in IM9 cells were examined by Western blot. (B) Analysis of luciferase reporter activity in IM9 cells after transfected with pNF-κB-luc or the control-luciferase plasmid. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with corresponding control group. (C) Cells were transfected with PSMB4 expression vector, and then treated with NF-κB inhibitor (Bay11-7082, 10 μM), the expression of miR-21 was analyzed by qRT-PCR. (D) The colony forming ability of IM9 cells was analyzed by soft agar colony-forming experiment. The number of colony was summarized in the bar graph and representative images were shown.

Previous studies demonstrated that elevated PSMB4 expression was associated with worse relapse-free survival (RFS) in breast cancer and decreased overall survival (OS) in ovarian cancer [14]. Our results demonstrated that PSMB4 contributed to the colony-formation and proliferation abilities of MM cells and might therefore represent a novel therapeutic target. Followed analysis showed that genetic inhibition of PSMB4 markedly decreased cell growth (Fig. 2), suggesting the potential therapeutic strategy against MM by targeting PSMB4.

It is well established that some microRNAs may function as oncogenes promoting cancer cell growth and tumorigenesis [27]. Previous researches have shown that miR-21 was up-regulated and positively related to MM progression and poor prognosis [19], and targeting miR-21 inhibited MM cell growth [28]. In our study, miR-21 was up-regulated in MM plasma, and in a public dataset, we also found that high PSMB4 expression was correlated with high miR-21 expression (Fig. 3B), suggesting positive correlation of PSMB4 with miR-21 expression in MM. Further analysis showed that ectopic PSMB4 triggered miR-21 expression, and PSMB4 depletion markedly repressed the level of miR-21 expression in MM cells, suggesting the regulation of miR-21 by PSMB4. Furthermore, while re-expression of miR-21 markedly rescued cell growth in PSMB4-deficient cells, decrease of miR-21 attenuated such events in PSMB4-overexpressing cells, indicating that PSMB4 promoted cell growth in part by potentiating miR-21 expression, providing new insights into potential mechanisms of MM development.

Recent observations elucidated that the proteolysis of many tumor suppressor proteins by proteasome was related to activation of several signing pathways, including NF-κB, resulting in increased proliferation of tumor cells [9,10]. It has been reported that NF-κB could be activated by proteasomes through degradation of the inhibitor of NF-κB (IκBα), mediating cancer cell survival and growth [29]. However, the roles of PMSB4 in regulation NF-κB activation are largely unknown. Our results suggested that PSMB4 exerted a profound impact on the activation of the NF-κB pathway. While overexpression of PSMB4 led to an increase in the activity of NF-κB and a decrease in the level of IκBα (Fig. 4), depletion of PSMB4 completely abrogated such events, suggesting PSMB4 may activates NF-κB signaling through a proteasome-mediated protein degradation of IκBα. Additionally, the expression of several microRNAs, including miR-21, could be regulated by transcription factor, such as NF-κB [20]. This prompted us to explore the possibility of PSMB4-mediated up-regulation of miR-21 by activating NF-κB. Indeed, blocking NF-κB markedly abolished PSMB4-induced miR-21 expression as well as the cell viability, indicating the regulation mechanism of PSMB4 in MM tumorigenesis via a NF-κB-miR-21 signaling pathway.

In conclusion, our results proposed a novel regulatory mechanism driving MM growth by PSMB4 via activation of the NF-κB signaling pathway, suggesting that PSMB4 functioned as an oncoprotein in MM progression and may serve as a novel therapeutic target for MM.

Conflict of interest

We declare no any interest conflict.

Acknowledgment

None.

Transparency document

The transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrc.2015.01.110.

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