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Promoter methylation induced epigenetic silencing of *DAZAP2,* a downstream effector of p38/MAPK pathway, in multiple myeloma cells

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ABSTRACT

Multiple myeloma (MM) is hematological malignancy characterized by clonal proliferation of malignant plasma cells in the bone marrow environment. Previously, we identified *DAZAP2* as a candidate cancer suppressor gene, the downregulation of which is regulated by its own promoter methylation status. In the current study, we analyzed the *DAZAP2* promoter in MM cell lines KM3, MM.1S, OPM-2, and ARH77 by bisulfite genomic sequencing assay. We identified the binding site for transcription factor cyclic adenosine monophosphate response element binding (CREB) in the *DAZAP2* promoter CpG2, and we found that hypermethylation of the *CREB* binding motif in the *DAZAP2* promoter is responsible for the reduced *DAZAP2* expression in MM cells. Later we checked the p38/MAPK signaling cascade, which is reported to regulate expression and function of CREB. Our results showed that the p38/MAPK signaling pathway drives the expression of *DAZAP2* by phosphorylation of CREB, and hypermethylation of *CREB* binding motif in *DAZAP2* promoter can inhibit binding of CREB to the latter, thus downregulating *DAZAP2* expression. Moreover, treating the MM cells with 5-aza-2′ deoxycytidine to demethylate *DAZAP2* promoter restored the binding of CREB to its binding motif, and thus upregulated *DAZAP2* expression. Our results not only identified *DAZAP2* as a new downstream target of p38/MAPK/CREB signaling cascade, but we also clarified that the downregulation of *DAZAP2* in MM cells is caused by hypermethylation of *CREB* binding motif in its own promoter region, which implies that demethylation of *DAZAP2* promoter can be a novel therapeutic strategy for MM treatment.

1. Introduction

Multiple myeloma (MM), also known as cancer of plasma cells, is characterized by clonal proliferation of malignant plasma cells in the bone marrow environment [1]. The pathogenesis of MM correlates with multiple changes in the bone marrow microenvironment [2], karyotypic instability [3], abnormality of signaling pathways [4], and production of cytokines such as osteoclast activating factors [5]. Benefitting from the rapid development of next-generation sequencing and large-scale analysis of patient' specimens, epigenetic deregulation was revealed as a critical factor in MM pathogenesis, and numerous molecules targeting epigenetic machinery have been developed and considered as potential therapeutic strategies for MM. DNA methylation, a key module of epigenetic regulation, is closely related to MM progression, so that a relationship between MM stage and methylation pattern was observed [6]. Promoter methylation of specific genes has been reported to play important roles in the therapeutic response in numerous tumors or cancer, including MM [7–9].

DAZAP2 (deleted in azoospermia-associated protein 2) was originally isolated in a mouse gene trap screen as a transcript expressed in the inner ear [10]; it was also known as the interacting partner of DAZ (deleted in azoospermia), an RNA-binding protein-encoding gene expressed in germ cells, which was associated with spermatogenesis. The *DAZAP2* is broadly expressed in mouse and human tissue and it plays diverse roles in cell biology and physiology. Our previous studies have shown that the significantly downregulated *DAZAP2* expression in MM cell lines was mediated by the methylation of specific regions in *DAZAP2* promoter [11–13]. Both CpG islands in the *DAZAP2* promoter exert synergistic effects on the regulation of the expression of *DAZAP2;* somehow the second island possesses higher transcriptional activity. Nevertheless, the transcription factors that drive *DAZAP2* transcription and the signaling pathways involved in mediating its gene expression remain to be explored.

The cyclic adenosine monophosphate (cAMP) response element binding (CREB) protein is a member of the basic leucine zipper (bZIP)-containing transcription factor family. CREB functions as a mediator of

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pleiotropic biological activities including cell proliferation and differentiation, survival, glucose homeostasis, DNA repair, angiogenesis, spermatogenesis, circadian rhythms, and immune responses [14]. The overexpression of CREB was observed in a number of solid tumors and hematopoietic malignancies, the downregulation of which resulted in the inhibition of cell proliferation, invasion and induction of apoptosis, suggesting it might be a potential therapeutic target for cancer [15]. The involvement of CREB in tumor progression has also been shown to be mainly mediated by aberrant activation of cAMP signal-related pathways, resulting in increased CREB activity via phosphorylation. Moreover, CREB has been observed to participate in the development of tumor resistance against inhibitors of several signal pathways [16,17].

In the current study, we hired bioinformatic methods to analyze the CpG islands of *DAZAP2* promoter, and identified binding sites of transcription factor CREB in the second CpG island of *DAZAP2* promoter. Next, we found that the downregulation of *DAZAP2* in MM cells is related with hypermethylation in the *CREB* binding motif in the *DAZAP2* promoter. The canonical p38/MAPK signaling pathway plays a vital role in mediating the expression of multiple transcription factors, and CREB is one of the downstream effectors of p38/MAPK pathway[18]. Herein, we showed that P38/MAPK signaling pathway drives the expression of *DAZAP2* by phosphorylation of CREB, and this expression can be inhibited by the hypermethylation of *CREB* binding motif in the *DAZAP2* promoter. Methylated *CREB* binding motif prevents the binding of CREB to *DAZAP2* promoter, inhibits further *DAZAP2* transcription, thus downregulating the expression of *DAZAP2,* and this inhibition can be restored by demethylation of *DAZAP2* promoter. Our study identified *DAZAP2* as a new downstream target of P38/MAPK/CREB signaling pathway, and the methylated *DAZAP2* promoter with a hypermethylation in the *CREB* binding motif is able to inhibit the expression of *DAZAP2*. Moreover, demethylation of *DAZAP2* promoter can be considered as a novel therapeutic strategy for MM.

2. Material and methods

2.1. Cell lines and cell culture

Human MM cell lines ARH-77, MM.1S were purchased from the American Type Culture Collection (ATCC; USA). Human MM cell line OPM-2 was from the Cell Center of Shanghai Chuan-Xiang Biotechnology (Shanghai, China). Human MM cell line KM3 was a gift from Professor Jian Hou from Shanghai Changzheng Hospital, Shanghai, China. Human immortalized B lymphocytes were gifts from the Center for Medical Genetics, School of Life Sciences, Central South University, China. ARH-77, MM.1S, KM3were cultured in RPMI 1640 (Life Technologies, USA), supplemented with 10% fetal bovine serum (FBS) (Biological Industries, USA), 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.OPM-2 was cultured in Dulbecco modified Eagle medium (Life Technologies), supplemented with 10% FBS (Biological Industries), 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The B lymphocytes were cultured in RPMI 1640 supplemented with 25% FBS. All the cells were cultured at 37 °C in the presence of 5% CO_2 in the humidified chamber.

2.2. Reagent and antibodies

The Magna ChIP™ A/G kit (Cat. #17-10,085) were purchased from Millipore (USA). Pierce™ ECL Plus Western Blotting Substrate Kit (32132) was purchased from Thermo Fisher Scientific™ (USA). 5-aza-2′ deoxycytidine was purchased from Sigma-Aldrich. The DNA Clean System was purchased from Promega. The CelLytic™ NuCLEAR™ Extraction Kit (NXTRACT) was purchased from Merck. The LightShift™ chemiluminescent electrophoretic mobility shift assay (EMSA) kit was purchased from Thermo Fisher Scientific. The small interfering (si) RNAs siB09917112221, siG000001385B, and siG14529165721were purchased from RiboBio, along with ribo*RECT*™CP. The TRIzol reagents were purchased from Life Technologies. The Revert Aid Reverse Transcriptase was purchased from Thermo Fisher Scientific™. The TB Green™ Premix Ex Taq™ was purchased from TaKaRa. Anti-CREB-1 (Cat. SC-186), anti-DAZAP2 (Cat. SC-515182), and anti-GAPDH (Cat. SC-25778) were purchased from Santa Cruz Biotechnology. Anti-phospho-p38 (Cat. #9215), anti-p38 (Cat. #9212), anti-phospho-CREB (Cat. #9198) , anti-phospho-MSK1(Cat. #9591), and anti-MSK1(Cat. #3489) were purchased from Cell Signaling Technology.

Table 1
The PCR primers used for ChIP assay.

Primer name	Sequence (5′ → 3′)	Fragment size (bp)
Primer-1-F	GAAAACACTTAATCGCTGCCCTA	193
Primer-1-R	ACGCAACACCTGGCACTGAAACT	
Primer-2-F	TTTCAGTGCCAGGTGTTGCGTAC	222
Primer-2-R	TCCCCACACCTTAGTTTATCATG	
Primer-3-F	TGATAAACTAAGGTGTGGGGAGC	224
Primer-3-R	GTCCCTCAGCTGGAGAAAAACCA	
Primer-4-F	TTTTTCTCCAGCTGAGGGACACC	252
Primer-4-R	TTGTCTCGGCGTCGCGGACGGTT	

2.3. Chromatin immunoprecipitation assay

Protein-DNA complexes were cross-linked by 1% formaldehyde, then quenched using 125 mM glycine. Cells were collected in lysis buffer and subjected to sonication. After centrifugation, the supernatant was incubated with CREB antibody, and chromatin DNA was purified by DNA Clean System. DNA was amplified by quantitative polymerase chain reaction (qPCR) and normalized to input. The primers for qPCR are listed in Table 1.

2.4. Electrophoretic mobility shift assay

The biotin-labeled methylated and unmethylated probes, unlabeled methylated and unmethylated probes were synthesized by the TaKaRa. The probe sequence for EMSA assay were listed in Table 2. The binding reactions were performed according to the manufacturer's instructions. The reaction mixture was incubated at 25 °C for 15 min, then samples were run on 6% native polyacrylamide gels in 0.5 × TBE buffer, followed by detection using electrochemiluminescence (ECL) assay.

2.5. RNA interference and overexpression

Transfection of siRNA was accomplished using the ribo*RECT*™CP according to the manufacturer's instruction. Cells were harvested for further analysis from 24 h to 72 h after transfection with the siRNA. For gene overexpression, recombinant plasmids were constructed and used for transfection. 2×10^5 cells were seed in 6-well plates and transfected with 2-μg plasmids. After 48 h, cells were harvested for further analysis.

2.6. PCR and real-time PCR

Total RNA was isolated using TRIzol regents according to the manufacturer's instructions and complementary DNA synthesis was carried out using Revert Aid Reverse Transcriptase. TB Green™ Premix Ex Taq™ was used for qPCR. The primers used for gene expression

Table 2
Probe sequence for EMSA assay.

Probe name	Sequence
Unmethylated probe	5′-GCG TCG GGT GAC GCT AGG CGG AC-3′ 3′-CGC AGC CCA CTG CGA TCC GCC TG-5′
Methylated probe	5′-GAG TTG GGT GAC GCT AGG CTG AC-3′ 5′-CTC AAC CCA CTG CGA TCC GAC TG-3′

Table 3
The primers used for quantitative PCR.

Primer name	Sequence (5′ → 3′)	Size (bp)
CREB-F	AAA GAC TTT TCT CCG GAA C	220
CREB-R	TAC AGT GGT GAT GGC AGG	
DAZAP2-F	ACA GCC AAC CTA CCC TGT GCA	210
DAZAP2-R	CAT GAC TGC AAG CTG AGC AGC	
ACTIN-F	TGA CGG TCA GGT CAT CAC TAT CGG CAA TGA	220
ACTIN-R	TTG ATC TTC ATG GTG ATA GGA GCG AGG GCA	@

analysis were listed in Table 3.

2.7. Western blot analysis

Cells were lysed in radioimmunoprecipitation assay buffer and proteins (25–35 μg) were separated using 8–10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride membranes (Millipore). After blocking membranes, they were incubated with appropriate dilutions of primary antibodies; horseradish peroxidase conjugated secondary antibodies, and visualized using the ECL system.

2.8. Statistics

All experiments were performed at least three times. All statistical analyses were carried out with SPSS 19.0. The data values were presented as the mean ± standard deviation. Differences in mean values between two groups were analyzed by two-tailed *t-t*-test and the mean values of more than two groups were compared with one-way analysis of variance. Significant differences are shown by an asterisk ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

3. Results

3.1. Methylation status of DAZAP2 promoter in MM cells and its effect on DAZAP2 expression

Our previous studies showed that the methylation of *DAZAP2* promoter resulted in the downregulation of *DAZAP2* expression in MM cell lines. There are two CpG islands in the *DAZAP2* promoter, of which CpG2 plays a more important role in regulating the expression of *DAZAP2*. Bioinformatics analysis using TFSEARCH revealed that there is a *CREB* binding site in the CpG2 sequence of *DAZAP2* promoter region. There are 23 CpG dinucleotide sequences in CpG2 of the *DAZAP2* promoter. Among them, two CpG dinucleotides (CG18 and CG19) are contained in the *CREB* binding motif (Fig. 1A). To investigate the role of transcription factor CREB in the regulation of *DAZAP2* expression, we analyzed the methylation status of *DAZAP2* promoter CpG2 in MM cell lines KM3, MM.1S, OPM-2, and ARH77 by bisulfite genomic sequencing assay. Our results indicated that the methylation of *CREB* binding motif in *DAZAP2* promoter CpG2 occurred at CG18 in KM3 and MM.1S cells, where KM3 showed a higher degree of methylation. No methylation of *CREB* binding motif occurred in OPM-2 and ARH77 cells (Fig. 1B).

In order to verify the correlation between CREB and DAZAP2 expression level in MM cells, we detected the expression of CREB and DAZAP2 in MM cells and human B lymphocytes at both the messenger RNA (mRNA) and protein level. The results showed that although there was no significant difference in the expression of CREB between MM cells and B cells, the expression of DAZAP2 was different in MM cells. The expression of DAZAP2 was normal in OPM-2 and ARH77 cells with an unmethylated *CREB* binding motif at mRNA level, and it was significantly downregulated in KM3 and MM.1S cells with a methylated *CREB* binding motif at both the mRNA and protein level (Fig. 1C, D). These results suggested that methylation of *CREB* binding motif in *DAZAP2* promoter might affect the binding of CREB to the promoter region of *DAZAP2*, resulting in downregulation of DAZAP2 expression in MM cells.

3.2. Methylation of CREB binding motif in DAZAP2 promoter inhibits the binding of CREB to DAZAP2 promoter

DNA methylation is an important epigenetic modification active in heterochromatin. Moreover, DNA methylation is the cause of the changes in chromatin structure, DNA conformation, DNA stability, and the way DNA interact with proteins, thus controlling gene expression. The CpG islands are typically located in the transcriptional regulatory region of the 5′ flank sequence of structural genes, and usually are in a nonmethylated state. Hypermethylation of CpG islands may lead to inhibition of gene transcription. To verify the effect of methylation of *CREB* binding motifs in *DAZAP2* promoter on the regulation of *DAZAP2* expression itself, chromatin immunoprecipitation (ChIP) and EMSA were carried out to evaluate the binding ability of CREB to the *DAZAP2* promoter.

We analyzed the binding ability of CREB to *DAZAP2* promoter region under different methylation status in KM3, KM3/5-AZA (we treated KM3 cells with 5 μmol/L 5-aza-2′ deoxycytidine for 72 h to demethylate the *DAZAP2* promoter), and B cells by conducting the ChIP assay. Four pairs of primers (P1-P4) were designed to amplify approximately 1000 bp upstream of *DAZAP2* promoter region. Among them, P3 amplified the sequence of CpG1 and P4 amplified the sequence of CpG2. The results indicated that CREB could bind to *DAZAP2* promoter CpG2 (P4) when the *CREB* binding motif was unmethylated (B cells representing the unmethylated status). When the *CREB* binding motif was methylated (KM3 cells representing the methylated status), CREB could not bind to *DAZAP2* promoter CpG2. When *CREB* binding motif was demethylated (KM3/5-AZA cells representing the demethylated status), not surprisingly, we found that CREB restored its binding to CpG2 (Fig. 2A).Therefore, when the *CREB* binding motif is under the unmethylated or demethylated state, the binding ability of CREB to *DAZAP2* promoter CpG2 is much higher than that of the methylated state (Fig. 2B).These results suggest that the methylation of *CREB* binding motif in *DAZAP2* promoter CpG2 could inhibit the binding of CREB to the *DAZAP2* promoter.

The 23-bp fragments containing methylated or unmethylated *CREB* binding motif were synthesized and used as probes for EMSA analysis. The EMSA results showed that CREB could bind to the unmethylated probes, but not to the methylated probes, and the methylated probes could not competitively inhibit this binding (Fig. 2C). Based on these results, we hypothesize that transcription factor CREB induces *DAZAP2* transcription by binding to the *DAZAP2* promoter region. When the *CREB* binding motif of *DAZAP2* promoter CpG2 was methylated, CREB could not bind to the *DAZAP2* promoter, thus inhibiting the transcription of *DAZAP2* (Fig. 2D).

3.3. Regulation of DAZAP2 expression by methylation status of CREB binding motif

In order to verify whether CREB was involved in the regulation of DAZAP2 expression, both RNA interference and overexpression were carried out to change the expression level of CREB. Then we detected DAZAP2 expression under different CREB expression levels. Our results showed that knockdown of CREB in B cells could effectively downregulate the expression of DAZAP2 (Fig. 3A), whereas the overexpression of CREB in B cells could upregulate the expression of DAZAP2 (Fig. 3B). However, when the *CREB* binding motif was under a methylated state (KM3 cells), overexpression of CREB had no effect on the expression of DAZAP2 in these cells (Fig. 3C). These results suggest that CREB may be a key transcription factor in the regulation of DAZAP2 expression in MM cells, and the hypermethylation of *CREB* binding motif in *DAZAP2* promoter inhibits the binding of CREB to *DAZAP2* promoter, thus resulting in a profound downregulation of

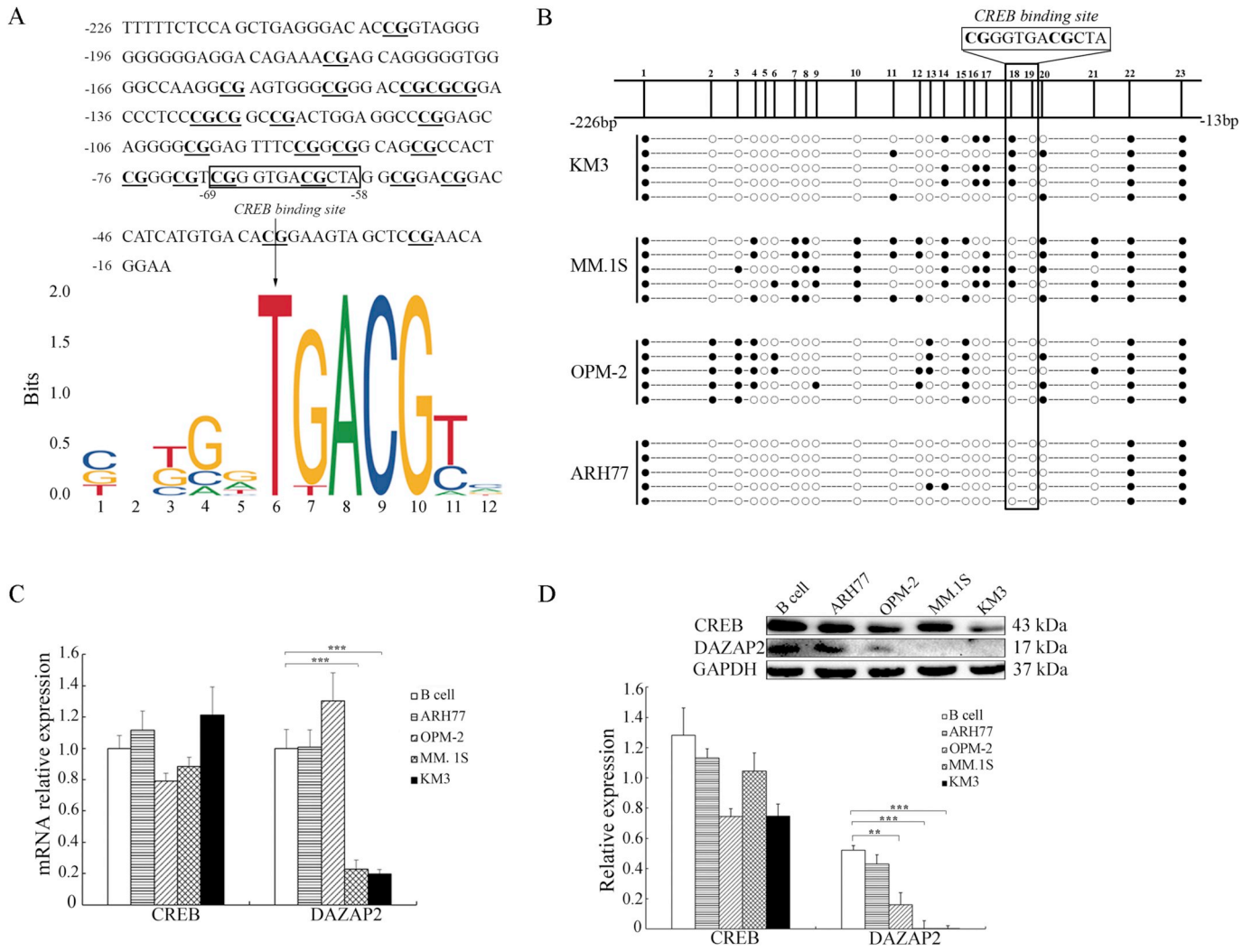

Fig. 1. Analysis of *DAZAP2* promoter methylation status and the correlation between CREB with *DAZAP2* expression.
(A) CpG dinucleotide sequences in CpG2 of the *DAZAP2* promoter, and *CREB* binding site in the CpG2 of *DAZAP2* promoter. (B) Analysis of the methylation status of different MM cell lines by bisulfite genomic sequencing assay. (C) Differences in *CREB* and *DAZAP2* at the mRNA level of MM cell lines and B cells. (D) Differences in CREB and DAZAP2 at the protein expression level of MM cell lines and B cells. Error bars indicate standard deviation, *** P < 0.001, ** P < 0.01.

DAZAP2 expression in MM cells.

3.4. Involvement of p38/MAPK pathway in regulation of DAZAP2 expression

CREB is known as one of the downstream effectors of p38/MAPK pathway. To further explore the upstream signaling cascade responsible for CREB-mediated *DAZAP2* expression in MM pathogenesis, we hypothesized that the p38/ MAPK signaling pathway was involved in the regulation of DAZAP2 expression in MM cells. In order to verify our hypothesis, both p38/MAPK activator anisomycin and inhibitor SB202190 were used to activate and suppress p38/MAPK signaling pathway in different MM cell lines and B cells. When the KM3 and MM.1 cells were treated with anisomycin, the p-p38, p38,p-CREB, and CREB were increased, and the expression of DAZAP2 had a slight increase in MM.1S but not in KM3 (Fig. 4A, 4B), the latter had a higher degree of methylation in the *CREB* binding motif. Moreover, in ARH77 (the *CREB* binding motif in ARH77 was unmethylated) and B cells with higher DAZAP2 expression, adding SB202190 could downregulate the expression of p-p38, p38, p-CREB, CREB, and DAZAP2 (Fig. 4C, D). These results suggested that p38/MAPK signaling pathway was involved in the regulation of DAZAP2 expression in MM cells by phosphorylation of CREB, and methylation in the *CREB* binding motif is involved in the reduction of DAZAP2 expression.

3.5. Methylated CREB binding motif inhibits the DAZAP2 expression driven by p38/MAPK signaling pathway

In order to elucidate the effect of methylated *CREB* binding motif on DAZAP2 expression driven by p38/MAPK signaling pathway, we used both anisomycin and SB202190 to detect the effect of activating or inhibiting p38/MAPK signaling pathway on the expression of *DAZAP2* under different methylation status.

In B cells, where *CREB* binding motif was under an unmethylated status, the expression of DAZAP2 was maintained at a high level. When B cells were treated with SB202190, the relative expression of p-p38/p38 ($n = 0.11 \pm 0.03$), p-CREB/CREB ($n = 0.24 \pm 0.10$) reduced significantly, and the expression of DAZAP2 was reduced. When anisomycin was added to the SB202190 treated B cells, the p38/MAPK signaling pathway was activated again as shown by the increased relative expression of p-p38/p38 ($n = 0.73 \pm 0.20$), p-CREB/CREB ($n = 1.08 \pm 0.14$). Not surprisingly, the expression of DAZAP2 recovered (Fig. 5A). These results indicate that p38/MAPK signaling pathway plays an important role in maintaining the normal expression of DAZAP2 in cells.

In KM3 cells, where the DAZAP2 expression was low due to the

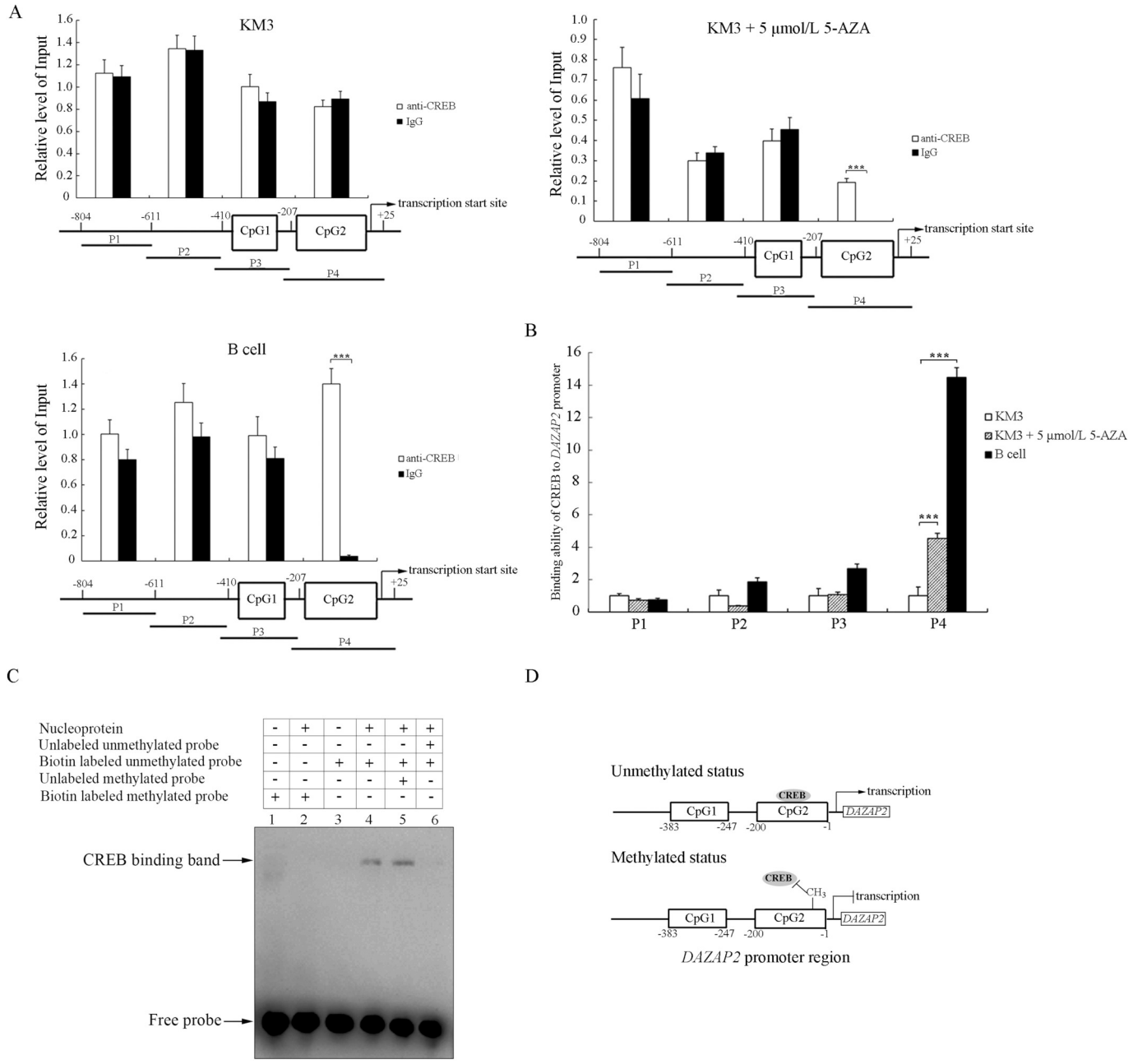

Fig. 2. Analysis of CREB binding to *DAZAP2* promoter by ChIP and EMSA assay.
(A) The binding of CREB to *DAZAP2* promoter P1, P2, P3, P4 in KM3 cells, KM3/5-AZA, and B cells. (B) The binding of CREB to *DAZAP2* promoter CpG2 under different methylation level. (C) The binding of CREB to *DAZAP2* promoter CpG2 by EMSA assay. Column 1: Adding only biotin labeled methylated probe; Column 2: Adding nucleoprotein with biotin labeled methylated probe; Column 3: Adding only biotin labeled unmethylated probe; Column 4: Adding nucleoprotein with biotin labeled unmethylated probe; Column 5: Adding nucleoprotein with biotin labeled unmethylated probe and unlabeled methylated probe; Column 6: Adding nucleoprotein with unlabeled unmethylated probe and biotin labeled unmethylated probe. (D) The schematic diagram of CREB binding to *DAZAP2* under methylated and unmethylated status. Error bars indicate standard deviation, *** $P < 0.001$.

hypermethylation of *CREB* binding motif in its own promoter, adding anisomycin profoundly upregulated the phosphorylation levels of p38, MSK1, and CREB in KM3 cells with a slight increase in the total protein of these molecules, as shown by the increased relative expression of p-p38/p38 ($n = 1.91 \pm 0.07$), p-MSK1/MSK1 ($n = 0.97 \pm 0.01$), p-CREB/CREB ($n = 1.95 \pm 0.05$). Somehow, the expression of DAZAP2 remained low. When SB202190 was added to the anisomycin-treated KM3 cells, the expression of phosphorylation level of P38 and CREB restored their expression to the level before the cells were treated with anisomycin, as shown by the decreased expression of p-p38/p38 ($n = 0.33 \pm 0.10$), p-CREB/CREB ($n = 0.39 \pm 0.04$), and the expression of DAZAP2 remained unchanged at a low level (Fig. 5B).

KM3/5-AZA cells were treated with SB202190 and anisomycin. The results showed that SB202190 could inhibit the phosphorylation of p38, MSK1 and CREB, as shown by the decreased expression of p-p38/p38 ($n = 0.32 \pm 0.07$), p-MSK1/MSK1 ($n = 0.04 \pm 0.01$), p-CREB/CREB ($n = 0.34 \pm 0.03$), thus down-regulating the expression of DAZAP2. When anisomycin was added to the SB202190 treated KM3/5-AZA, the inhibition can be reversed, as shown by the decreased expression of p-p38/p38 ($n = 0.50 \pm 0.04$), p-MSK1/MSK1 ($n = 1.12 \pm 0.03$), p-CREB/CREB ($n = 0.65 \pm 0.08$), and the expression of DAZAP2 was significantly increased (Fig. 5C).

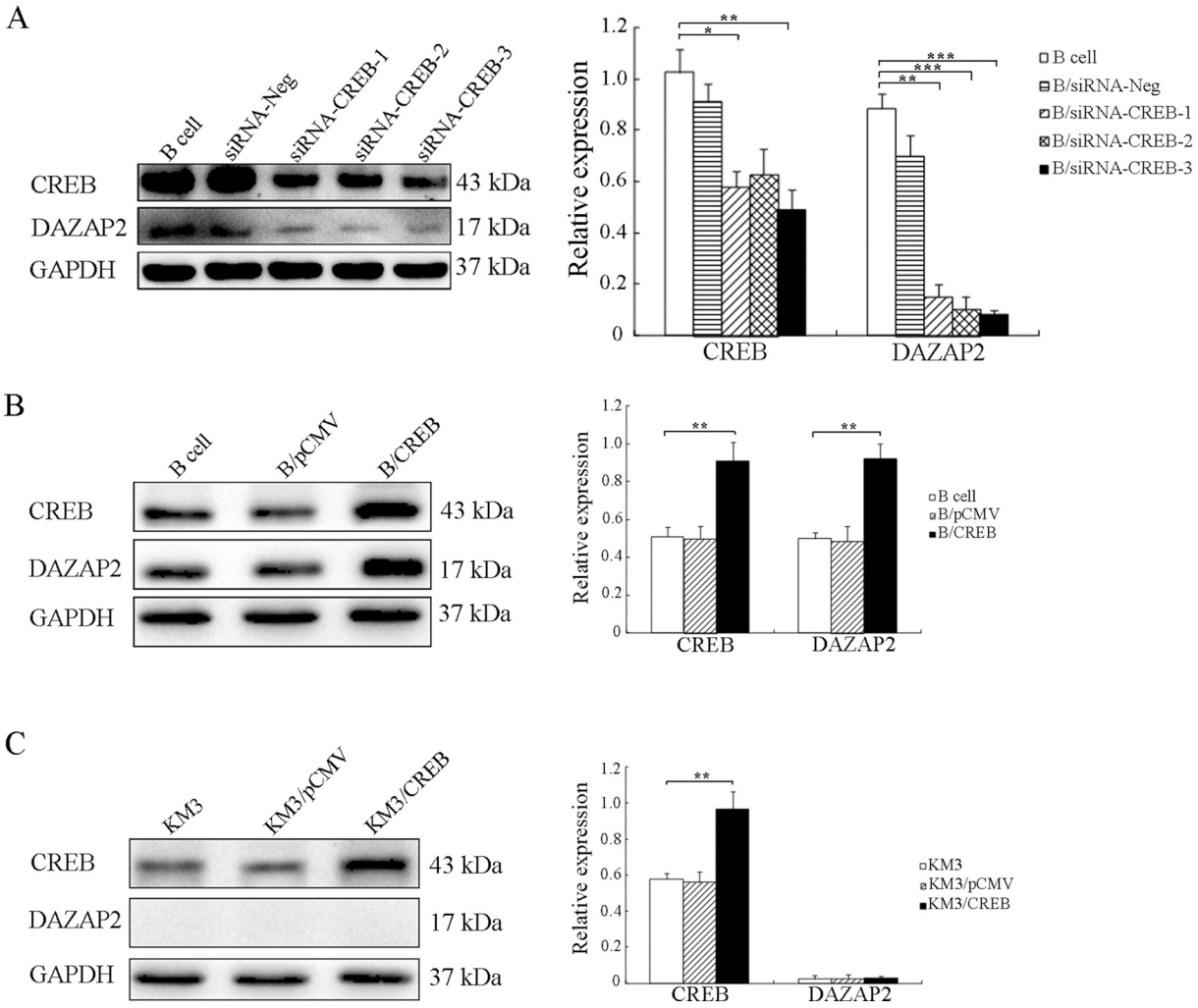

Fig. 3. Regulation of DAZAP2 expression by transcription factor CREB.
(A) Immunoblotting of DAZAP2 and CREB after knockdown of CREB in B cells. (B) Immunoblotting of DAZAP2 and CREB after overexpressing CREB in B cells. (C) Immunoblotting of DAZAP2 and CREB after overexpressing CREB in KM3. Error bars indicate standard deviation, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.
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These results suggest that the canonical p38/MAPK signaling pathway was involved in regulating the expression of DAZAP2 by phosphorylating its downstream effector CREB. However, the hypermethylated *CREB* binding motif in *DAZAP2* promoter disabled the binding of transcription factor CREB to the *DAZAP2* promoter, leading to the downregulated DAZAP2 in MM cells by blocking the P38/MAPK signaling cascade regulating DAZAP2 expression. The inhibition of DAZAP2 transcription by methylated *CREB* binding motif can be rescued by demethylation of *DAZAP2* promoter (Fig. 6).

4. Discussion

MM is an incurable malignancy of plasma cells due to its poorly understood pathogenesis. Over the past decade, results from large-scale whole-exome sequencing studies have shed new light on the clonal heterogeneity and evolution of the disease, and yield new insights into MM not anticipated by existing knowledge [19–22]. Epigenetic regulations as well as other posttranslational modifications of histone have been reviewed as key factors in the regulation of MM [23,24]. The hypermethylation of numerous known tumor suppressor genes has been shown to have correlations with MM, especially by genome-wide analysis [25–27]. The DNA methylation profile of MM is related to progression of the disease and certain classes of mutations in epigenetic modifiers are more prevalent upon disease relapse, suggesting a role in MM progression [28].

DAZAP2 was discovered to be broadly expressed during mouse embryonic development and in adult mouse and human tissues. Previous studies in our group have described the structure, expression, and molecular features of *DAZAP2* in the mononuclear cells from MM, that expression of *DAZAP2* is profoundly downregulated in MM [11–13]. Later, we found two CpG islands in the *DAZAP2* promoter region; these promoter regions were hypermethylated and suppressed *DAZAP2* expression in the MM cells [11]. In the current study, we identified a *CREB* binding site in the CpG2 island of *DAZAP2* promoter region in MM cells, and found that the hypermethylated *CREB* binding motif in the *DAZAP2* promoter is responsible for the reduced *DAZAP2* expression in MM cells. The hypermethylated *CREB* binding motif of *DAZAP2* promoter disabled CREB binding to the *DAZAP2* promoter, thus inhibit the transcription of *DAZAP2*, the demethylation can restore its binding.

CREB had been reported to act as a pleiotropic element in various physiological and pathological processes. Overactivated and constitutively phosphorylated CREB played a role in a number of malignancies, and thus appears to play a direct role in disease pathogenesis and prognosis [15,29]. Moreover, CREB has been reported to participate in B cell malignancies including MM. Regulation of CREB stability

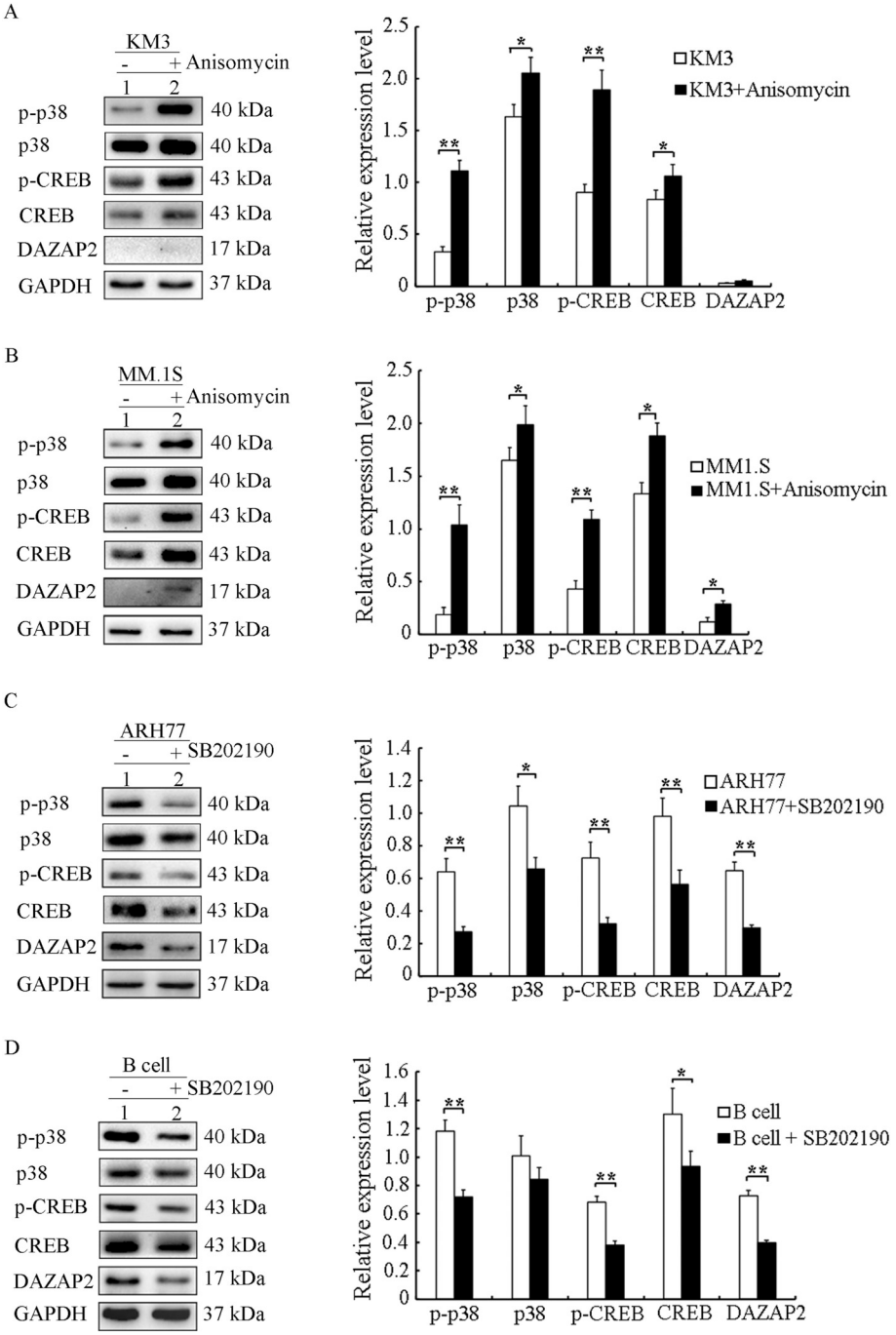

Fig. 4. Regulation of P38/MAPK signaling pathway in the expression of DAZAP2.
(A) Immunoblotting for p-P38, P38, p-CREB, CREB, and DAZAP2 after treating KM3 with anisomycin. (B) Immunoblotting for p-P38, P38, p- CREB, CREB, and DAZAP2 after treating MM.1S with anisomycin. (C) Immunoblotting for p-P38, P38, p-CREB, CREB, and DAZAP2 after treating ARH77 with SB202190. (D) Immunoblotting for p-P38, P38, p-CREB, CREB, and DAZAP2 after treating B cells with SB202190. Error bars indicate standard deviation, ** $P < 0.01$, * $P < .05$.

by the adaptor protein tumor necrosis factor receptor-associated factor 3 (TRAF3) is important for B cell homeostatic survival, and deregulation of TRAF3 is an important pathological factor for B cell malignancies [30]. CREB also had been reported as a downstream target of the canonical P38/MAPK pathway [18,31], and p38/MSK/CREB cascade mediates ATRA-induced Ape/Ref-1 expression and acquired chemoresistance in myeloma cells [32].

In order to investigate the correlation between *DAZAP2* expressions and p38/MAPK/CREB signaling cascade, we used p38/MAPK inhibitor SB202190 and activator anisomycin to treat the MM cells and B cells. ARH77 with an unmethylated *CREB* binding motif showed a similar decrease in p-p38, p-CREB, and DAZAP2 expression by responding to SB202190 compared to B cells; the latter represented the unmethylated status. Therefore, the p38/MAPK pathway drives the *DAZAP2* expression by phosphorylation of CREB. MM cell lines with discrepancies in the methylation status of *CREB* binding site showed significant difference in response to anisomycin. In MM.1S and KM3 cells, where the expression of DAZAP2 was low due to methylation in the *CREB* binding motif, MM.1S showed a slight increase in DAZAP2 expression compared to KM3, where KM3 had a higher degree of methylation. To further elucidate whether methylation status of *CREB* binding site is responsible for the reduced *DAZAP2* expression in MM cells, we used 5-

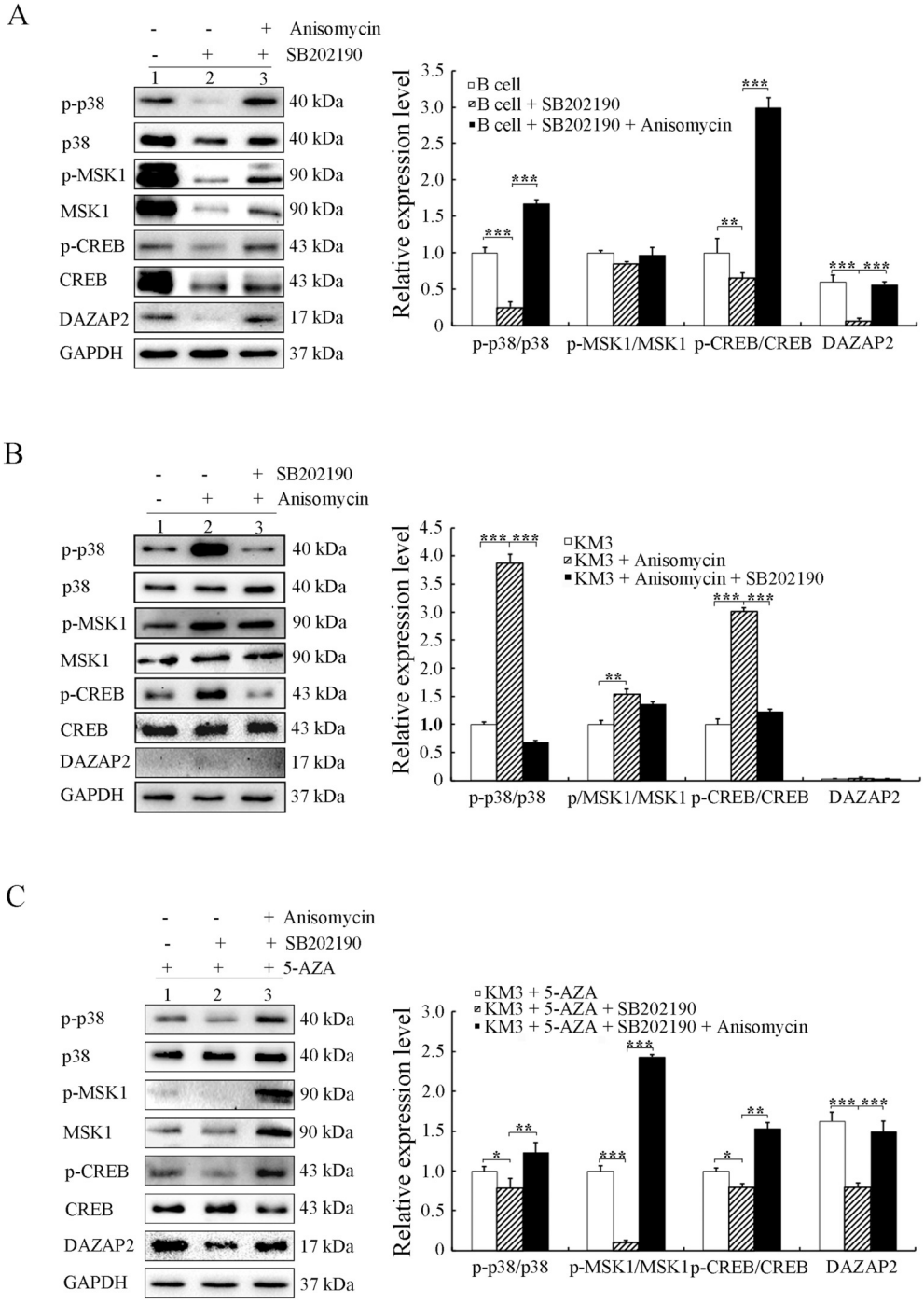

Fig. 5. Effect of methylated *CREB* binding motif on the expression of DAZAP2 driven by P38/MAPK pathway.
(A) Immunoblotting of p-p38, p38, p-MSK1, MSK1, p-CREB, CREB, and DAZAP2 after treating B cells with SB202190 and SB202190+ anisomycin. (B) Immunoblotting of p-p38, p38, p-MSK1, MSK1, p-CREB, CREB, and DAZAP2 after treating KM3 with anisomycin and anisomycin+SB202190. (C) Immunoblotting of p-p38, p38, p-MSK1, MSK1, p-CREB, CREB, and DAZAP2 after treating KM3/5-AZA with SB202190 and SB202190+ anisomycin. Error bars indicate standard deviation, *** $P < 0.001$, ** $P < 0.01$.

aza-2′ deoxycytidine to demethylate the *DAZAP2* promoter, and compared DAZAP2 expression in KM3 (hypermethylated status), B cells (unmethylated status), and KM3/5-AZA (demethylated status) in response to anisomycin and SB202190. We found that DAZAP2 expression KM3 cells fail to respond to P38/MAPK pathway activator and inhibitor, where B cells and KM3/5AZA showed similar response to P38/MAPK pathway activator and inhibitor in expression of DAZAP2.

DAZAP2 is a candidate tumor suppressor gene in MM driven by the P38/MAPK signaling pathway. The methylated *CREB* binding motif disabled CREB binding to *DAZAP2* promoter and resulted in the reduced expression of *DAZAP2*. Our findings not only revealed *DAZAP2* as a new downstream target of P38/MAPK signaling cascade, but also provided more choices for development of targeting therapies, especially for those patients with severe drug resistance. DAZAP2 has been reported to have an association with Wnt signaling pathway [33], and P38/MAPK is not the only signaling pathway that regulates CREB [15]. For example, PI3K-dependent CREB activation is a factor in aromatase-induced tamoxifen-resistant breast cancer [17].

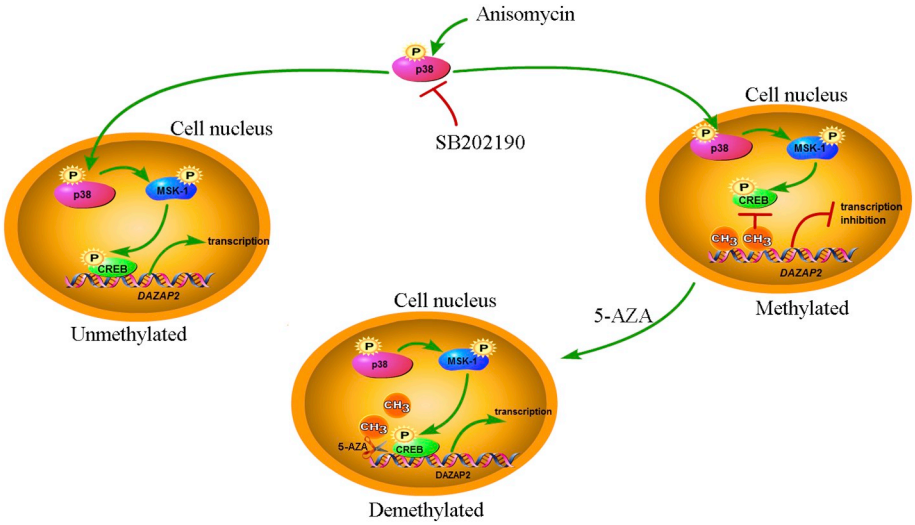

Fig. 6. Schematic diagram of interaction between DAZAP2 methylation and P38/MAPK signaling pathway.

Therefore, whether the downregulation of DAZAP2 in MM cells is regulated by signaling pathways other than P38/MAPK/CREB merits further investigation. Moreover, CREB has also been reported to participate in osteoclastogenesis, which is an important pathological factor in myeloma bone disease (MBD). Whether hypermethylation of *CREB* binding motif in *DAZAP2* promoter has a correlation with osteoclastogenesis in MBD and whether this can be a therapeutic target in MBD deserves further study.

The current paradigm of *DAZAP2* has been focused on its downregulation in the MM cells. The association of *DAZAP2* promoter with transcription factor CREB not only advanced our understanding of *DAZAP2* in its role as a candidate tumor suppressor gene in MM, but also expanded our knowledge of novel therapeutic strategies for MM, such as the development of specific targeted drugs against this methylation site.

Author contributions

JH, WXH and JL conceived and wrote the manuscript. JL, SQL DHX, SS, YPW, XFB. JH designed and performed the experiments. JH, WXH, JL and SQL evaluated and analyzed the results.

Conflict of interest

The authors declare no potential conflicts of interest.

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