Article

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EZH2 Promotes Multiple Myeloma Progression via STAT3 Pathway Activation

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Background: Multiple myeloma (MM) is a malignant disorder of plasma cells in the bone marrow. MM causes the clonal proliferation of terminally differentiated plasma cells and the accumulation of monoclonal plasma cells. The enhancer of zeste homolog 2 (EZH2) has been proven to play a significant role in disease development and could act on the signal transducers and activators of the transcription 3 (STAT3) signaling pathway. This pathway contributes to the pathogenesis and maintenance of malignancies. This study aimed to explore the effect of EZH2 on MM progression and the role of the STAT3 pathway in this process. The goal was to increase knowledge and provide further insights about the pathogenesis of MM and identify novel targets for potential therapies.

Methods: The abnormal expression of EZH2 in MM cell lines was tested through real-time reverse transcriptase-polymerase chain reaction (RT-qPCR) and western blot analysis. Based on the MM cell line H929, transfection was used to modify EZH2 expression, followed by the subsequent evaluation of induced alteration in STAT3 activation. The STAT3 phosphorylation activator colivelin and inhibitor stattic were used for promoting and inhibiting the STAT3 activation, respectively. Colony-forming assay, transwell migration assay, and flow cytometry were used to explore cell proliferation, cell migration, and cell apoptosis, respectively.

Results: Both the EZH2 mRNA and protein were over-expressed in multiple MM cell lines including H929 ($p < 0.001$), U266 ($p < 0.01$), RPMI-8226 ($p < 0.01$) and MM.1S ($p < 0.001$). Increased EZH2 promoted cell proliferation ($p < 0.001$) and migration ($p < 0.001$) and simultaneously inhibited cell apoptosis ($p < 0.001$), which could be reversed by inhibited STAT3 activation ($p < 0.001$). In contrast, promoted STAT3 activation increased cell proliferation ($p < 0.001$) and migration ($p < 0.001$), while simultaneously inhibiting cell apoptosis ($p < 0.001$), despite decreased EZH2 expression.

Conclusions: The effect of EZH2 and STAT3 pathways on MM regulation was revealed and verified. EZH2 promoted the progression of MM cells by activating the STAT3 pathway. The EZH2 and STAT3 pathways could be potential targets for effective MM treatment.

Keywords: multiple myeloma; EZH2; STAT3

Introduction

Multiple myeloma (MM), which accounts for 10% of all hematologic malignancies [1], is a malignant disorder of plasma cells in bone marrow, resulting in the clonal proliferation of terminally differentiated plasma cells and the accumulation of monoclonal plasma cells [2]. MM is characterized by the overproduction of monoclonal proteins (M proteins), a type of abnormal immunoglobulin, by malignant myeloma cells [3]. The overproduction of M proteins causes bone lesions or renal insufficiency, increasing vulnerability to conditions like anemia, infections, or hypercalcemia [4]. The survival rate of MM patients has doubled in the past decade due to the introduction of new, less toxic, and more effective therapeutic strategies [5,6]. However, the long-term sustainability of treatment-related progress is unclear since nearly all patients eventually relapse and the efficacy of available therapies remains elusive [7]. Given the volatility of MM and the likelihood of relapse, increased knowledge and further pathogenic insights for novel treatments are necessary.

The enhancer of zeste homolog 2 (EZH2), the enzymatic subunit of the polycomb repressive complex 2 (PRC2), functions significantly in both normal development and disease processes [2]. EZH2 functions as a histone methyltransferase that contributes to the regulation of gene expression involved in cell self-renewal, the cell cycle, cell proliferation, and cell differentiation [8,9]. EZH2 also functions as a non-histone methyltransferase which is independent of the PRC2 complex [10,11] and methylates non-histone proteins such as signal transducers and activators of transcription 3 (STAT3), promoting tumorigenicity proteins [12]. Moreover, STATs have been recognized as major contributors to cell functions such as proliferation, angiogenesis, metastasis, survival, and invasion [13,14]. Addition-

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ally, STAT proteins play roles in hematopoiesis [15] and the dysregulation of STAT pathways takes part in the pathogenesis of malignancies [16]. STAT3 and STAT5 are two specific STAT proteins that are implicated strongly in the pathogenesis of cancers [17] while EZH2 has been shown to expand cancer stem cells and maintain malignancies via the STAT3 signaling pathway [18,19]. However, the relationship between the STAT3 pathway and EZH2 on MM progression remains unclear.

In the present study, we verified EZH2 overexpression in MM cells and exploited the MM cell line H929 as the experimental model. We investigated how EZH2 and the STAT3 pathway affect MM cells by analyzing changes in cell proliferation, cell migration, and cell apoptosis. The present study initially proved the significance of STAT3 on EZH2 function and consequently on MM progression, providing a deeper understanding of MM and its potential treatments.

Materials and Methods

Cell Culture and Treatment

The human B lymphocyte cell line GM12878 (iCell-h406) and human MM cell lines H929 (iCell-h311), U266 (iCell-h266), RPMI-8226 (iCell-h183), and MM.1S (iCell-h291) were purchased from iCell Bioscience Inc. (Shanghai, China) and were cultured in RPMI-1640 medium (iCell-0002, iCell Bioscience Inc., Shanghai, China) containing 10% fetal bovine serum (FBS) (iCell-0500, iCell Bioscience Inc., Shanghai, China) and 1% penicillin/streptomycin (iCell-15140-122, iCell Bioscience Inc., Shanghai, China). All cell cultures were stored at 37 °C in an atmosphere of 5% CO_2 and 70–80% humidity. All cell lines underwent mycoplasma testing and short tandem repeats (STR) identification confirmed that no cross-contamination occurred in the cell lines. To activate STAT3 signaling via increased STAT3 phosphorylation, cells received 6 h treatment with 0.5 µM colivelin (HY-P1061A, MedChemExpress, Shanghai, China) [20]. To inhibit STAT3 signaling by decreasing STAT3 phosphorylation, cells were treated with 3 µM Stattic (HY-13818, MedChemExpress, Shanghai, China) for 24 hours [21].

Transfection

Small interfering RNAs against EZH2 (si-EZH2; 5′-ACU CAU UGG UUC CUU UAA GGG-3′, 5′-CUU AAA GGA ACC AAU GAG UCC-3′) and negative control (si-NC) were purchased from Guangzhou Ribo Biotechnology Co., Ltd. (Guangzhou, China). The H929 cells were seeded at 100 × 10^5 cells/well in 12 well plates and transfected with 10 pM siRNA. The reagent Lipofectamine™ 2000 (11668019, Thermo Fisher Scientific, Shanghai, China) was utilized to perform the transfection according to the manufacturer's instructions. Before extracting RNAs, transfected cells were incubated at 37 °C for 48 h and the medium of transfected cells needed to be replaced 24 h after transfection.

In addition, Lentivirus containing pcDNA-EZH2 (OE-EZH2) and the empty pcDNA3.0 vector (OE-NC) were obtained from Shanghai Genechem Co., Ltd. (Shanghai, China). The transfection was performed following the manufacturers' instructions, and puromycin (2.0 µg/mL) was used to screen transfected cells for one week.

Western Blot

To conduct western blot analysis, proteins were extracted from cells, and the supernatant containing the total protein was collected. The total protein concentrations in both whole tissue and cell extracts were measured using the BCA Protein Quantitation kit (P0012, Beyotime, Shanghai, China). The loading buffer was added at 1/4 of the total protein solution volume. Cell lysis was then denatured at 100 °C in boiling water for 15 minutes to prepare for subsequent SDS-PAGE electrophoresis. Afterwards, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (IPVH00010, Millipore Sigma, Billerica, MA, USA) which were blocked with 5% skim milk (G5002, Serveicebio, Wuhan, China) at room temperature for 1 h, and incubated with primary antibodies (EZH2: 1:1000 dilution, 21800-1-AP, Proteintech, Wuhan, China; STAT3: 1:2000 dilution, 28445-1-AP, Proteintech, Wuhan, China; p-STAT3: 1:1000 dilution, MA5-15193, Invitrogen, Carlsbad, CA, USA; GAPDH: 1:1000 dilution, TA-08, ZSGB-Bio, Beijing, China) at 4 °C overnight. The membranes were washed with PBSTw blocking buffer (P0222, Beyotime, Shanghai, China) for 15 min followed by incubation with the secondary antibodies (horseradish peroxidase labeled goat anti-mouse IgG: 1:5000 dilution, Cat. No. ZB-2305, ZSGB-Bio, Beijing, China; horseradish peroxidase labeled goat anti-rabbit IgG: 1:5000 dilution, Cat. No. ZB-2301, ZSGB-Bio, Beijing, China) at room temperature for 40 min. After a 15-minute PBST wash, the bands were visualized using the enhanced chemiluminescent (ECL; P0018AS, Beyotime, Shanghai, China) method, with GAPDH serving as the internal reference.

Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-qPCR)

For RT-qPCR analysis, total RNAs were extracted from cells or tissues using TRIzol® (DP424, Tiangen, Beijing, China). RNA purity was assessed using a full-wavelength spectrophotometer (mµLtiskan sky, Thermo Fisher Scientific, Shanghai, China) with a desired ratio of OD260 nm/OD280 nm falling within the range of 1.8–2.2. RNAs were reverse transcribed into cDNAs using a reverse transcription kit (KR116, Tiangen, Beijing, China) per the manufacturer's instruction. The qPCR was conducted using a fluorescence quantitative instrument for PCR (CFX connect, Bio-Rad Laboratories (Shanghai) Co., Ltd., Shang-

hai, China), employing a specific cycling program of thermal conditions. Relative expression levels were normalized to the geometric mean of expression of the housekeeping gene Actin and the $2^{-\Delta\Delta Cq}$ method was used for processing the data. The primer sequences used in this study were as follows: EZH2-F, 5′-TTG TTG GCG GAA GCG TGT AAA ATC-3′; EZH2-R, 5′-TCC CTA GTC CCG CGC AAT GAG C-3′; GAPDH-F, 5′-ATG GAG AAG GCT GGG GCT C-3′; GAPDH-R, 5′-AAG TTG TCA TGG ATG ACC TTG-3′.

Colony Forming Assay

Transfected cells were initially left untreated for 48 hours. Then, cells in the exponential growth period were trypsinized and dispensed into 6-well dishes for tissue culturing with a density of 1000 counts/well. Cells were cultured for 14 days before they were fixed with 1 mL 4% paraformaldehyde (P0099, Beyotime, Shanghai, China) and stained with Giemsa (G1064, Solarbio, Beijing, China) for visualization.

Transwell Migration Assay

Migration assay was performed using the transwell kit (3422, Corning Inc., Corning, NY, USA). Cells were initially left untreated for 48 h and then, the suspension (100 µL) of 1×10^5 cells with no serum in the medium was seeded into the upper chamber. Simultaneously, a complete medium (600 µL) with 30% FBS was added to the lower chamber. Following a 24-hour incubation at 37 °C, the nonmetastatic cells present on the top surface of the membrane were removed. Subsequently, the metastatic cells located on the lower surface of the membrane were stained with 0.1% Giemsa for 15 minutes. Subsequently, metastatic cells were examined with an inverted fluorescent microscope (CKX53, OLYMPUS, Tokyo, Japan) at 100× magnification. Three random fields were photographed to count cells, and the average number of migrated cells was used to quantify migration capacity.

Apoptosis Analysis

Transfected H929 cells were cultured for 24 h and harvested by trypsinization without EDTA. After being washed twice with cold PBS and collected following centrifugation, cells were re-suspended in the 1× binding buffer to a concentration of 1×10^6 cells/mL. Afterward, 100 µL cell suspension containing 1×10^5 cells was stained with 5 µL Annexin V-PE and 5 µL 7-AAD (Annexin V-PE/7-AAD apoptosis kit; MA0429, Meilunebio, Dalian, China) at room temperature for 15 min. The staining operations were fulfilled in dark conditions and cell apoptosis was measured via a flow cytometer (NovoCyte, Agilent Technologies Co., Ltd., Beijing, China). Results were analyzed by CellQuest and visualized by a two-color dot plot.

Statistical Analyses

Experimental data from experiments conducted repeatedly were presented as the mean ± standard deviation. The data of all experiments were analyzed through GraphPad Prism 8.0.2 software (GraphPad Software Inc., San Diego, CA, USA). Statistical analyses were performed using analysis of variance (ANOVA) followed by Tukey's post hoc test. The threshold of $p < 0.05$ was considered statistically significant.

Results

EZH2 Overexpression in MM Cells Promotes Activation of the STAT3 Pathway

The results of RT-qPCR (Fig. 1A) and western blot (Fig. 1B) showed significant overexpression of EZH2 in MM cells, evident in both mRNA levels ($p < 0.001$) and protein levels ($p < 0.01$). The results in Fig. 1C,D demonstrated that the si-EZH2 transfection decreased EZH2 protein expression ($p < 0.001$) and STAT3 pathway activation (by decreasing p-STAT3/STAT3; $p < 0.001$). Similarly, OE-EZH2 transfection increased EZH2 protein expression ($p < 0.001$) and STAT3 pathway activation (by increasing p-STAT3/STAT3; $p < 0.001$). Moreover, as shown in Fig. 1E, si-EZH2 transfection decreased EZH2 mRNA expression ($p < 0.01$) and OE-EZH2 transfection increased EZH2 mRNA expression ($p < 0.001$), while the STAT3 pathway activator colivelin and inhibitor stattic did not alter EZH2 mRNA expression ($p > 0.05$).

EZH2 Promotes H929 Cells Progression

As shown in Fig. 2A, cells in the si-EZH2 group formed fewer colonies compared with cells in the si-NC group ($p < 0.001$) while cells in the OE-EZH2 group formed more colonies compared with cells in the OE-NC group ($p < 0.001$). The results of the transwell migration assay (Fig. 2B) revealed that si-EZH2 transfected cells migrated less ($p < 0.001$) while OE-EZH2 transfected cells migrated more ($p < 0.001$) compared with normal H929 cells. The apoptosis results (Fig. 2C) revealed that more cancer cell apoptosis was induced in si-EZH2 transfected cells ($p < 0.001$) while less cancer cell apoptosis was induced in OE-EZH2 transfected cells ($p < 0.001$). Accordingly, EZH2 promoted H929 cell proliferation and migration while suppressing apoptosis.

EZH2 Functions on Cell Progression via STAT3 Pathway

As shown in Fig. 3A, OE-EZH2 increased EZH2 protein expression ($p < 0.001$) and increased STAT3 pathway activation (by increasing p-STAT3/STAT3; $p < 0.001$). Conversely, the STAT3 pathway inhibitor, stattic, did not affect EZH2 protein expression ($p > 0.05$) but decreased STAT3 pathway activation (by decreasing p-STAT3/STAT3; $p < 0.001$). Stattic significantly de-

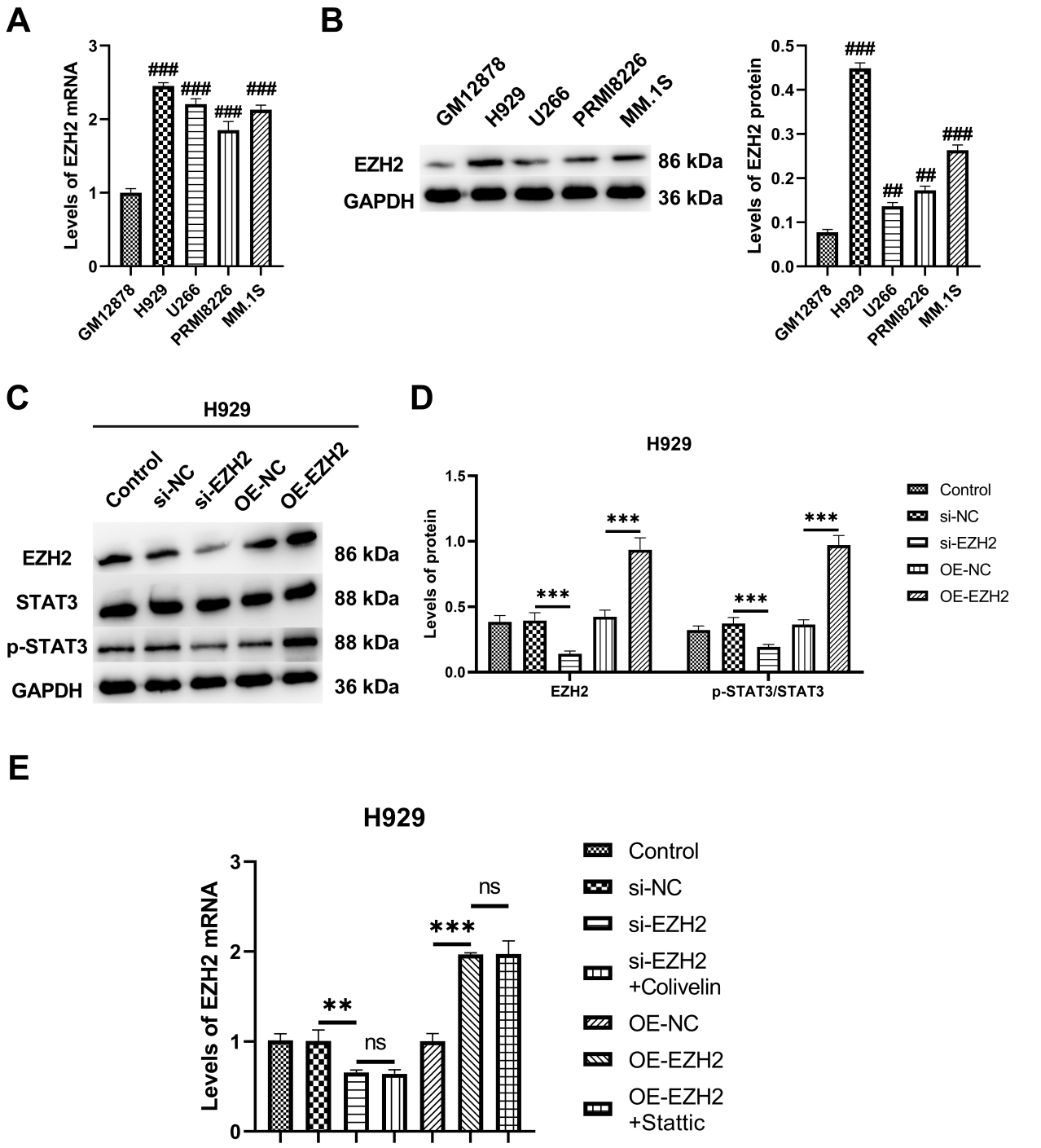

Fig. 1. The enhancer of zeste homolog 2 (EZH2) was overexpressed in multiple myeloma (MM) cells and promoted signal transducers and activators of transcription 3 (STAT3) pathway activation. The expressions of (A) EZH2 mRNA (n = 3) and (B) EZH2 protein (n = 3) in human B lymphocyte GM12878 cells and human multiple myeloma cells H929, U266, RPMI-8226 and MM.1S. The (C) representative western blot reactive images and (D) the quantification (n = 3) of EZH2, p-STAT3/STAT3. (E) The levels of EZH2 mRNA in transfected H929 cells (n = 3). Colivelin, the STAT3 signaling activator; Stattic, the STAT3 signaling inhibitor. ##$p < 0.01$, ###$p < 0.001$ vs. GM12878 group. **$p < 0.01$, ***$p < 0.001$, ns $p > 0.05$ (not significant).

creased the increased colony formation of OE-EZH2 transfected H929 cells (Fig. 3B; $p < 0.001$). Furthermore, in OE-EZH2 transfected H929 cells, the increased cell migration (Fig. 3C; $p < 0.001$) and decreased cell apoptosis (Fig. 3D; $p < 0.001$) were all reversed by the inhibition of STAT3 signaling (migration, $p < 0.001$; apoptosis, $p < 0.001$). Additionally, as shown in Fig. 4A, si-EZH2 decreased EZH2 protein expression ($p < 0.001$)

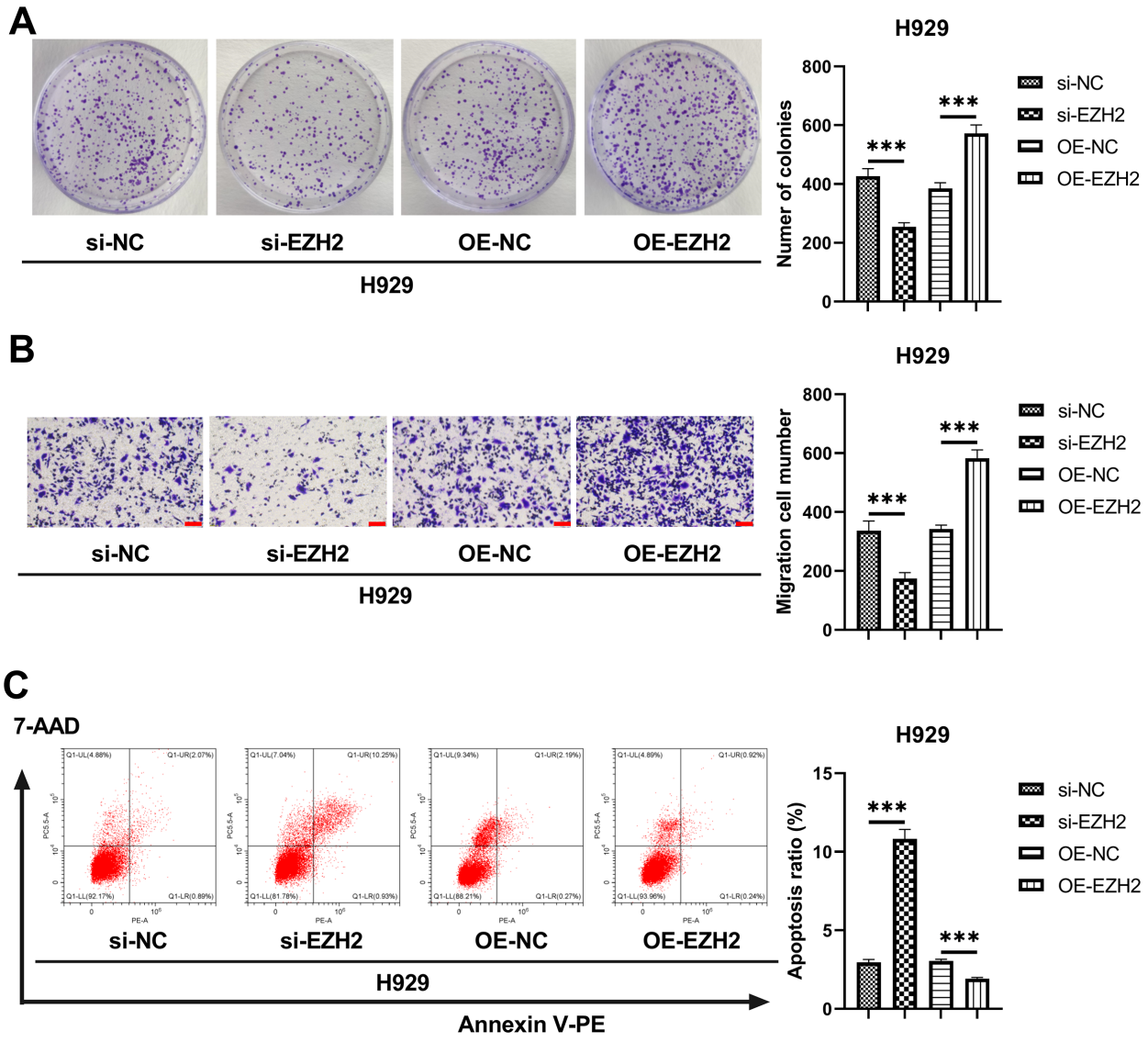

Fig. 2. EZH2 promotes the progression of H929 cells. (A) The results of colony-forming assay (n = 3). (B) The results of transwell migration assay (n = 4; scale bar: 50 µm). (C) The results of flow cytometry after transfected cancer cells were double stained (n = 3). ***$p < 0.001$.

and decreased STAT3 pathway activation (by decreasing p-STAT3/STAT3; $p < 0.001$), whereas the STAT3 pathway activator, colivelin, did not affect EZH2 protein expression ($p > 0.05$) but increased STAT3 pathway activation (by increasing p-STAT3/STAT3; $p < 0.001$). The decreased colony formation of si-EZH2 transfected H929 cells (Fig. 4B; $p < 0.001$) increased by stattic (Fig. 4B; $p < 0.001$). Furthermore, in si-EZH2 transfected H929 cells, the decreased cell migration (Fig. 4C; $p < 0.001$) and increased cell apoptosis (Fig. 4D; $p < 0.001$) were all reversed by the activation of STAT3 signaling activation (migration, $p < 0.001$; apoptosis, $p < 0.001$). All of these findings suggest that EZH2 contributed to MM cell progression via STAT3 pathway activation.

Discussion

Previous researches have reported the overexpression of EZH2 without mutation in MM [22–24]. Additionally, despite the heterogeneity observed in MM, bone marrow cells isolated from patients with MM or MM cell lines displayed constitutive activation and up-regulation of STAT3, while no mutations were observed [25,26]. These findings support that EZH2 was related to MM oncogenesis through STAT3 pathway activation, enabling EZH2 and STAT3 as potential MM treatment targets.

A previous meta-analysis showed that the high expression of EZH2 was significantly associated with the poor survival of patients with various solid malignancies [27]. Analyses of public datasets from clinical trials revealed that symptomatic MM patients expressed higher EZH2 mRNA

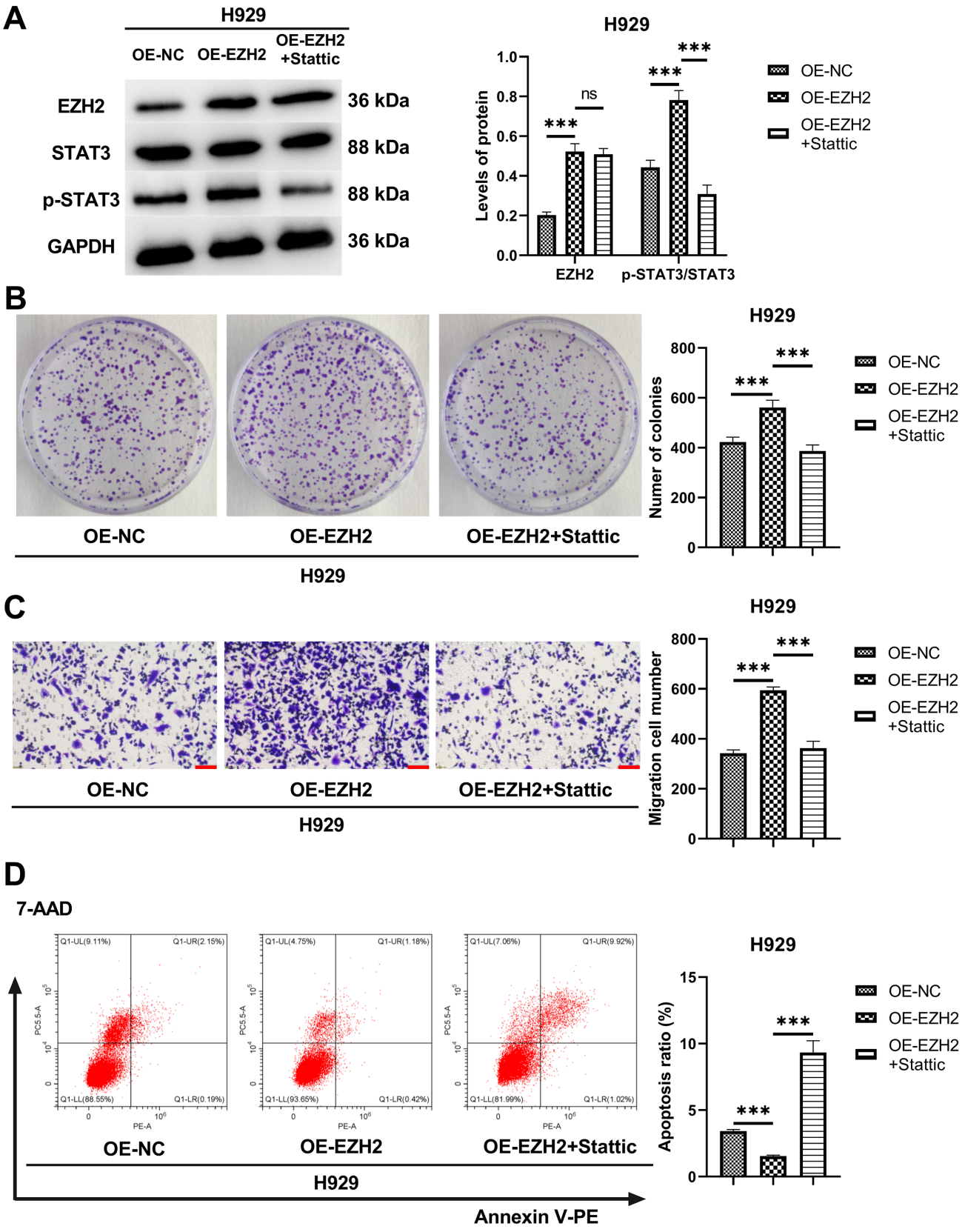

Fig. 3. EZH2 functions on cell progression via the STAT3 pathway. (A) The western blot results (n = 3). (B) The result of colony-forming assay (n = 3). (C) The result of transwell migration assay (n = 4; scale bar: 50 μm). (D) The result of flow cytometry after transfected cancer cells were double stained (n = 3). ***$p < 0.001$, ns $p > 0.05$ (not significant).

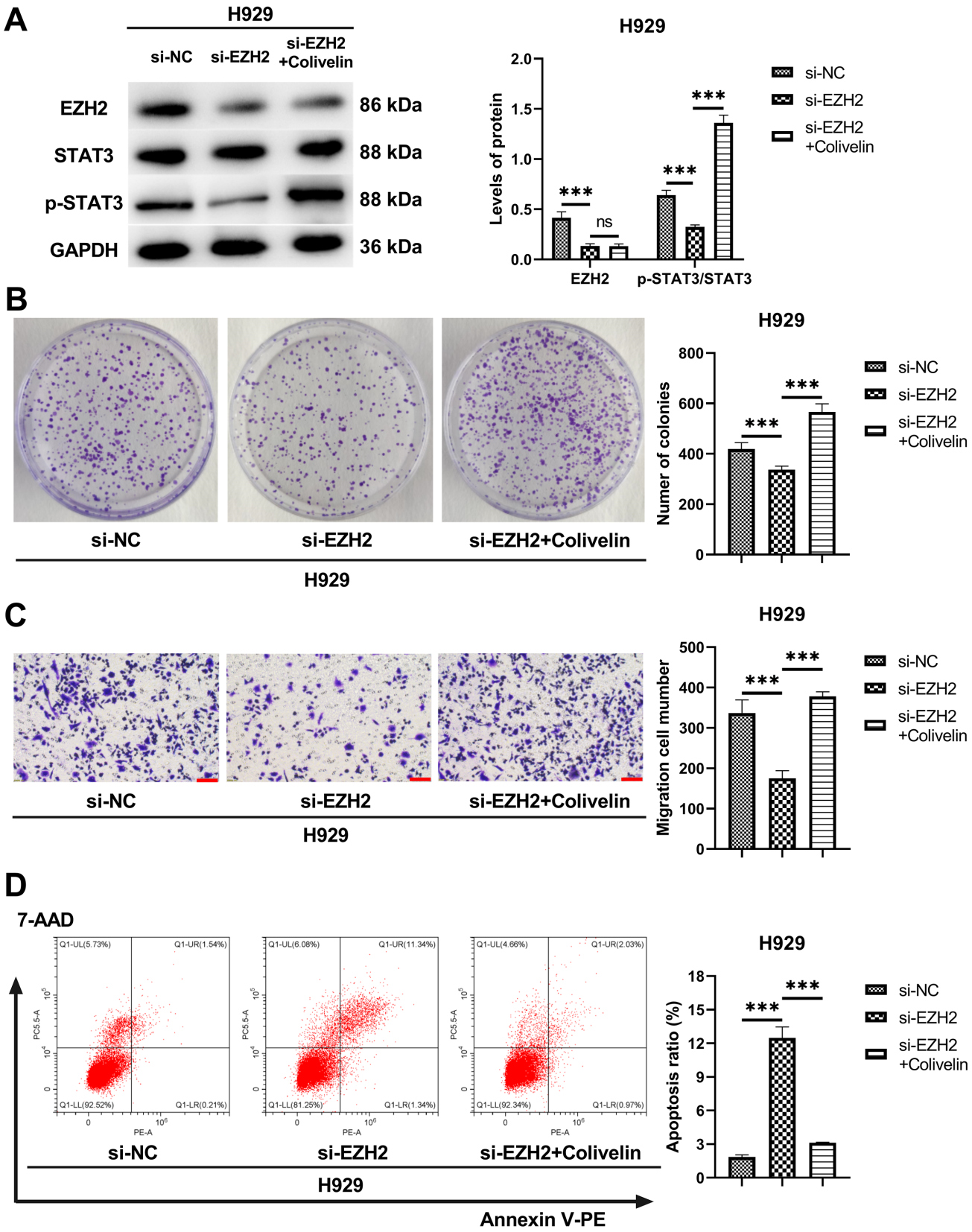

Fig. 4. EZH2 functions on cell progression via STAT3 pathway. (A) The western blot results (n = 3). (B) The result of colony-forming assay (n = 3). (C) The result of transwell migration assay (n = 4; scale bar: 50 μm). (D) The result of flow cytometry after transfected cancer cells were double stained (n = 3). ***$p < 0.001$, ns $p > 0.05$ (not significant).

than smoldering MM patients [28], establishing EZH2 as an independent prognostic factor associated with a decreased survival rate. Bioinformatics analyses found that EZH2 expression was upregulated in relapsed MM patients and was associated with disease promotion [29]. The current study validated the role of EZH2 in advancing MM cell progression, correlating with a poorer prognosis. This was evident through increased cell proliferation and migration, coupled with decreased cell apoptosis observed in H929 cells transfected with OE-EZH2 to up-regulate EZH2 expression. Conversely, H929 cells transfected with si-EZH2 to down-regulate EZH2 possessed decreased cell proliferation and migration, alongside increased cell apoptosis. These findings align with previous studies that EZH2 inhibition resulted in decreased cell line proliferation and tumor progression in mouse models [30].

EZH2-induced STAT3 pathway activation was identified as a mechanism by which disease progressed in various cancers. The methylating and activating role of EZH2 on STAT3 was proven to exacerbate breast cancer [31] and promote tumorigenicity of glioblastoma stem-like cells [32]. Additionally, the activation of EZH2 and the STAT3 pathway in breast cancer was implicated as a factor inducing acquired chemoresistance [18]. STAT3 has been shown to regulate the growth of head and neck squamous cell cancer (HNSCC), operating in an EZH2-dependent manner [33]. Additionally, EZH2's involvement in STAT3 pathway activation has been reported to contribute to neuroendocrine differentiation of prostate cancer [34]. In the present study, we observed the co-occurrence of EZH2 over-expression/under-expression and STAT3 pathway promoted/inhibited activation. Moreover, our observations revealed a decrease in MM cell proliferation and migration, coupled with increased apoptosis when inhibiting the activation of the STAT3 pathway, despite the up-regulation of EZH2 expression. This suggests that EZH2's impact on MM progression likely occurs through the activation of STAT3. The findings above support that the EZH2 played roles in the pathogenesis and progression of MM via STAT3 pathway activation, which has been proven as an unfavorable prognosis factor in MM, shortening the survival of patients and promoting drug resistance [35]. In this study, despite the down-regulated EZH2 expression, the increased STAT3 could promote MM cell proliferation and migration while protecting cells from apoptosis, sustaining MM as a solid malignancy.

Conclusions

The present study revealed and verified the effect of EZH2 and the STAT3 pathway as MM regulators. EZH2 promoted the progression of MM through STAT3 pathway activation and was associated with the deterioration of MM. This study proved that EZH2 and the STAT3 pathway as potential targets for effective MM treatment.

Availability of Data and Materials

Data to support the findings of this study are available on reasonable request from the corresponding author.

Author Contributions

YW and HL contributed to the concept and designed the research study. DH and YG performed the research. LL and AW provided help and advice on the experiments. YW and JT contributed to the analysis and interpretation of the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

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