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Targeted Knockdown of Macrophage Migration Inhibitory Factor Enhances UVB Irradiation-Induced Apoptosis Via Increasing ROS Generation in Oral Squamous Cell Carcinoma

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Abstract

Objectives: We investigated the effects of macrophage migration inhibitory factor (MIF) knockdown or overexpression combined with ultraviolet radiation B (UVB) irradiation on cell proliferation and apoptosis of oral squamous cell carcinoma (OSCC). **Methods:** MIF expression in OSCC and adjacent tissues was detected by immunohistochemistry. MIF expression in human immortalized oral epithelial cells (HIOEC) and OSCC cells was detected by western blotting. MIF was knocked down or overexpressed in OSCC cell lines (SCC-25 and CAL-27). OSCC cells were set up into control (CON), MIF overexpression/knockdown (oeMIF/shMIF), CON + UVB, and oeMIF + UVB/shMIF + UVB groups based on their exposure to UVB irradiation. Cell line proliferation was studied using a cell counting kit-8 (CCK-8) and colony formation assays. Flow cytometry was applied for determination of apoptosis, cell cycle, reactive oxygen species (ROS) abundance, and mitochondrial membrane potential. Apoptosis-related proteins were assayed by western blotting. **Results:** The expression of MIF was significantly higher in OSCC tissues and cell lines than in adjacent tissues and HIOEC. MIF knockdown accompanied by UVB irradiation significantly hampered cell viability and proliferation compared to MIF knockdown or UVB irradiation alone. Western blotting and flow cytometry showed that MIF knockdown combined with UVB irradiation not only induced apoptosis via the mitochondrial pathway but also mediated the cell cycle. Flow cytometry showed that ROS and mitochondrial membrane potential depolarization were increased in the combination treatment groups compared with the mono-treatment groups. Additionally, the ROS scavenger N-acetylcysteine significantly attenuated MIF knockdown combined with UVB irradiation-induced apoptosis and reversed MIF knockdown combined with UVB irradiation-induced MAPK activation. **Conclusion:** MIF knockdown combined with UVB irradiation significantly inhibited the proliferation of OSCC cells. MIF was involved in UVB-induced ROS generation and enhanced UVB irradiation-induced mitochondria-dependent apoptosis of OSCC cells by activating the MAPK pathway. This suggests that MIF-targeted therapy combined with UVB irradiation may be a novel approach for treating OSCC.

Keywords

macrophage migration inhibitory factor, ultraviolet radiation B irradiation, oral squamous cell carcinoma, reactive oxygen species, apoptosis

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Abbreviations

MIF, macrophage migration inhibitory factor; UVB, ultraviolet radiation B; OSCC, oral squamous cell carcinoma; CCK-8, cell counting kit-8; ROS, reactive oxygen species; NAC, N-acetylcysteine; FBS, fetal bovine serum; PBS, phosphate buffer saline; OD, optical density; MMP, mitochondrial membrane potential; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; HRP, horseradish peroxidase

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Introduction

Oral cancer has become one of the most prevalent malignancies, with oral squamous cell carcinoma (OSCC) accounting for over 90% of them.[1] Over 350 000 patients diagnosed with OSCC are added to the total case number worldwide per year, and over half of them die of OSCC annually.[2] Surgery, with or without radiotherapy, remains the gold standard for OSCC treatment. However, current treatment regimens have serious side effects, including surgical treatment that damages adjacent tissues when removing tumor tissue, resulting in impaired eating and speaking ability[3]; complications such as oral mucositis in patients after radiation therapy[4]; and systemic adverse effects such as nausea/vomiting and anemia with most chemotherapeutic agents.[5] Therefore, it is important to identify adjunctive OSCC treatments.

From the functional point of view, ultraviolet (UV) radiation can be partitioned into UVA, UVB, and UVC.[6] UVB irradiation of cells can cause apoptosis by forming DNA photoproducts and reactive oxygen species (ROS), further generating DNA damage. ROS are extremely toxic, with an extremely short half-life, and can affect neighboring cells without affecting surrounding tissues.[7,8] UVB irradiation is widely used in treating both common and rare dermatological diseases such as atopic dermatitis, vitiligo, and scleroderma.[9]

The application of UVB irradiation has shown merit in tumor research. Granados-López et al showed that in cervical cancer cells, UVB treatment could inhibit proliferation and the cell cycle, aside from inducing apoptosis via p53 and the microtubule systems, suggesting that UVB is a possible cancer treatment option.[10] Sarkar et al found that anti-VEGF drugs enhance UVB irradiation-induced apoptosis in breast cancer cells.[8] Shi et al found that vitamin K3 plus UVB treatment not only inhibited skin squamous cell proliferation but also induced apoptosis in vitro, while it inhibited tumor growth and promoted apoptosis in vivo.[11] Recently, with the emergence of photosensitizers and UV sensitizers, which confer advantages of high specific selectivity to tumor tissues and low toxicity, photodynamic therapy has become an effective method for tumor diagnosis and treatment and has been successfully applied to treat early-stage cancers of the oral cavity, pharynx, and larynx.[12] However, the effect of UVB irradiation on OSCC has not been studied.

Macrophage migration inhibitory factor (MIF) is a multifunctional cytokine involved in the regulation of several physiological and pathological functions of the body, and it plays an important role in inflammatory and immune responses. In recent decades, numerous studies have reported high expression levels of MIF in various tumors, including gastric cancer, nasopharyngeal cancer, lung cancer, and squamous carcinoma, MIF is involved in tumor cell proliferation, differentiation, angiogenesis, and tumorigenesis, and it promotes tumor cell infiltration and distant metastasis.[13] Several studies have shown that MIF is highly expressed in OSCC. MIF expression is closely related to the development and metastatic invasion of OSCC, which are important factors in the prognosis of OSCC patients.[14,15] Zeng et al used small interfering RNA to knock down MIF gene expression and found that MIF knockdown inhibited proliferation, migration, and colony formation in OSCC cells.[16] MIF also plays an important role in UVB-induced tumor development and progression. Nagarajan et al found that pretreatment with a MIF antagonist (CPSI-1306) reduced UVB light-induced inflammation and squamous carcinogenesis.[17] Similar findings were obtained by Honda et al, who showed that MIF inhibited UVB-induced photodamage by blocking the expression of the apoptosis-regulated genes p53, bax, and p21.[18] Therefore, molecularly targeted therapy for MIF combined with UVB irradiation is an interesting and promising approach to improve therapeutic effects against OSCC.

In this study, OSCC cells were represented by the SCC-25 and CAL-27 cell lines to investigate the effects of MIF knockdown or overexpression combined with UVB irradiation on OSCC cell proliferation and apoptosis. Mechanistically, MIF knockdown combined with UVB irradiation may induce apoptosis in OSCC cells via ROS accumulation and MAPK pathway activation. Our results suggest that combining MIF molecular targeting therapy with irradiation (UVB) can inhibit OSCC proliferation and promote apoptosis at the cellular level and may be a new therapeutic approach for OSCC.

Materials and Methods

Sample Collection

OSCC and adjacent tissues were collected from OSCC patients admitted to Renmin Hospital of Wuhan University from July to November 2022. A total of 4 OSCC tissues and 4 adjacent tissues were collected for this study. All patients and their families signed an informed consent form.

Cell Culture and Lentivirus Transfection

The human tongue squamous carcinoma cell lines SCC-25 and CAL-27 possess different levels of malignancy. Both these lines have been adopted to study OSCC worldwide. Both cell

lines cultured in this study were derived from Cell Lines of the Chinese Academy of Sciences (Shanghai, China), cultured in Dulbecco's modified Eagle medium (DMEM, C11995500BT; Gibco) supplemented with 10% fetal bovine serum (FBS, A3160802; Gibco) and 1% penicillin–streptomycin solution (BL505A; Biosharp), and kept in a humidified environment with 5% CO_2 at 37 °C for culturing.

MIF-knockdown cells (shMIF) were established for further use. The human MIF short hairpin sequence was combined with lentiviral vector Hu6-MCS-CBh-IRES-Puromycin for stable transfection. Recombinant lentivirus was obtained from GeneChem (Shanghai, China). Puromycin at 0.5 mg/mL was added for the selection of cell lines expressing repressed MIF or containing an empty vector, which was further confirmed by western blotting.

oeMIF was generated for further use. The human MIF-coding sequence was combined with the lentiviral vector Ubi-MCS-3FLAG-CBh-IRES-puromycin. Recombinant lentivirus was obtained from GeneChem. Puromycin at 0.5 mg/mL was added for the selection of cell lines expressing MIF or containing an empty vector, which was further confirmed by western blotting. Untreated SCC-25 and CAL-27 cells were used as the control.

UVB Irradiation

A UVB lamp (Philips, The Netherlands) was used for irradiation, and UVB radiation illuminance was measured by a UV power meter (LS123 UV Power Meter, China). The UVB irradiation intensity was set at 10 $\mu W/cm^2$, with the irradiation dose determined by the exposure time. Before irradiation, the culture medium was washed away and replaced by the same volume of phosphate-buffered saline (PBS) to avoid UVB reduction caused by the medium contents. After UVB irradiation, the medium was re-added for further culturing. OSCC cells were then collected for analysis at the indicated time points post UVB irradiation. Control cells were treated in the same manner, without any irradiation.

Immunohistochemistry (IHC)

Tissue sections were prepared according to standard procedures for paraffin embedding. Sections were placed in 0.1 mol/L citrate buffer (pH = 6) at 100 °C and microwave boiled for 15 min. The samples were cooled naturally at room temperature, rinsed twice in PBS, and pre-incubated in goat serum blocking solution at 37 °C for 30 min. Subsequently, they were rinsed in PBS and incubated overnight at 4 °C with rabbit anti-MIF monoclonal antibody (1:400;20415-1-AP; Proteintech), then rinsed in PBS and incubated with secondary antibody solution. Then, a 500-fold dilution of final drops was stained with DAB solution, re-stained with hematoxylin, divided with alcohol in hydrochloric acid, and sealed. Five fields of view were randomly selected for observation under a microscope at 100× and 400× magnification. The tissue sections were graded and scored according to the intensity of the dye color, where no color was negative, light yellow was weakly positive, light brown was moderately positive, and brown was strongly positive.

CCK-8 Assay

The influence of MIF knockdown together with UVB irradiation on the viability of SCC-25 and CAL-27 cells was studied with the cell counting kit-8 (CCK-8) assay. OSCC cells were divided into negative control, shMIF, UVB, and shMIF + UVB groups before they were plated in 96-well plates (Constar, USA) with an original density of 1×10^4 cells per well. Then, the UVB and shMIF + UVB groups were treated with UVB at doses 0, 1, 2.5, 5, 7.5, 10, 12.5, and 15 mJ/cm^2, respectively, while the other 2 groups were left untreated. After 24 h of incubation, 10 μL per well of CCK-8 solution (G4103-5ML; Servicebio) was added before another 2 h incubation. Optical density (OD) values at the 450 nm wavelength were measured using a multi-mode plate reader (PerkinElmer, Singapore).

Colony Formation Assay

Colony formation assays for SCC-25 and CAL-27 cells were carried out. We prepared 4 groups of cells: CON, shMIF, UVB, and shMIF + UVB. Briefly, OSCC cells were seeded at the same original density of 3000 cells per well, followed by 2-week culture in 10% FBS medium. After being rinsed with PBS, the cells were incubated in 4% paraformaldehyde for 15 min and subjected to crystal violet staining for 30 min. After PBS washing and air drying, colonies were counted in 4×10 columns. The experiments were repeated thrice or more before image analysis with ImageJ software.

Flow Cytometry

The Annexin V PE/7-AAD staining kit (559763; BD Biosciences) was used for apoptosis detection. OSCC cells were seeded at an original density of 5×10^5 cells/well in 6-well plates before being grouped and processed as described above. The cells were then collected and rinsed with PBS, resuspended in 500 μL binding buffer for Annexin-V PE and 7-AAD staining, and incubated in the dark for 10 min. The apoptosis rate was measured by flow cytometry (Becton, Dickinson and Company, USA).

Cell cycle distribution detecting was conducted using a cell cycle kit (CCS012; Multi Sciences). The cells were seeded, grouped, and processed in the same way as for the CCK-8 assay before being collected and frozen overnight in 70% ethanol at 4 °C. Cells were then placed in PBS (0.01 M) solution containing 100 μL RNase A and incubated at 37 °C for 30 min with propidium iodide (50 μg/mL) before being incubated for 30 min in the dark at 4 °C. DNA content analysis was performed on a flow cytometer (Becton, Dickinson and Company).

A ROS assay kit (S0033S; Beyotime) was used to measure the abundance of ROS in the cells, which were seeded at the same density as before. After the cells were grouped and processed according to the CCK-8 assay, they were incubated with DCFH-DA. Free DCFH-DA was removed by serum-free medium after incubation. Finally, flow cytometry (Becton, Dickinson and Company) was applied for ROS detection.

A JC-1 assay kit (C2006; Beyotime) was used for mitochondrial membrane potential (MMP) determination. Cells were seeded at 5×10^5/well before grouping, and treatments were performed according to the CCK-8 assay. JC-1 dye staining solution was supplemented after the cells were collected, followed by 20-min incubation in the dark. After that, cells were centrifuged at 1000 rpm for 5 min, rinsed, and resuspended in 500 μL of 1× culture buffer. Fluorescence detection was accomplished by flow cytometry, with the parameters set as 488 nm excitation and 525 nm emission.

Western Blotting

The cells were collected and washed thrice with precooled PBS, lysed on ice with radioimmunoprecipitation analysis (RIPA; G2002-30ML; Servicebio) buffer, sonicated for 30 s, and lysed for 15 min on ice. A bicinchoninic acid protein assay kit (P0010; Beyotime) was bought for concentration determination. Ten microliters of each sample was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis before being transferred to polyvinylidene fluoride membranes (IPVH00010; Millipore). The membranes were incubated overnight at 4 °C with the corresponding primary antibodies, including anti-MIF (1:1000;20415-1-AP; Proteintech), anti-Bcl-2 (1:1000; 4223S; CST), anti-Bax (1:1000; 41162S; CST), anti-PARP (1:1000; 9532 T; CST), anti-caspase-3 (1:1000; 14220 T; CST), anti-cleaved-caspase-3 (1:1000; 9664 T; CST), anti-caspase-7 (1:1000; 12827 T; CST), anti-cleaved-caspase-7 (1:1000; 8438 T; CST), anti-P38 MAPK (1:1000; 8690 T; CST), anti-phospho-P38 MAPK(1:1000; 4511 T; CST), anti-JNK 1/2 (1:1000; 9252 T; CST), anti-phospho-JNK 1/2 (1:1000; 4668 T; CST), and anti-Flag-Tag (1:1000; 20543-1-AP Proteintech), as well as the internal reference glyceraldehyde-phosphate dehydrogenase (1:1000; GB11002; Servicebio). Next, the membranes were rinsed and blocked before being incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10000; BL003A; Biosharp) for 2 h. Signal transduction was performed using an ECL kit (BL520A; Biosharp), and a ChemiDocTM imaging system (Bio-Rad Laboratories, USA) was adopted for imaging.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, USA). Numbers are presented as mean ± SD of at least 3 separate experiments. In this study, results were checked by Student's *t*-test, and differences were considered statistically significant at $P < .05$.

Results

Expression of MIF in OSCC Tissues and OSCC Cell Lines

Compared with the human immortalized oral epithelial cells group, the expression of MIF in SCC-9, SCC-25, and CAL-27 was significantly increased ($P < .05$) (Figure 1A). Immunohistochemical results showed that the OSCC tissue was dark brown and strongly positive, whereas the adjacent tissue was colorless and negative (Figure 1B). In the collection of 4 samples, the MIF was positive in all OSCC tissues and negative in adjacent tissues.

Effect of UVB Irradiation on the Viability of OSCC Cells

The effect of UVB irradiation on the viability of OSCC cells was assessed in vitro. Each group of cells was treated with UVB doses of 0, 1, 2.5, 5, 7.5, 10, 12.5, and 15 mJ/cm^2, and cell viability was measured 24 h post irradiation. Cell viability declined in a dose-dependent manner by UVB. The IC_{50} values of UVB-irradiated CON groups were 5.36 and 8.71 mJ/cm^2 in SCC-25 and CAL-27 cells, respectively, while in the UVB-irradiated shMIF group, IC_{50} values were 4.225 and 7.01 mJ/cm^2, respectively (Figure 2A).

MIF Knockdown Combined With UVB Irradiation Inhibits Cell Proliferation in OSCC

The anti-proliferative effect of UVB irradiation on SCC-25 and CAL-27 cells was confirmed by clone formation experiments. The number of colonies declined significantly in cells that underwent MIF knockdown combined with UVB irradiation compared to MIF knockdown or UVB irradiation alone (Figure 2B).

MIF Knockdown Combined With UVB Irradiation Induces G2/M Phase Cell Cycle Arrest and Apoptosis in OSCC

To explore whether the proliferation inhibitory effect of MIF knockdown combined with UVB irradiation was mediated by cell cycle arrest, flow cytometry was applied for detecting the cell cycle distribution of each group. As demonstrated in Figure 3A, the proportion of cells at the G2/M phase was boosted significantly 24 h post UVB irradiation. The G2/M phase proportions of the shMIF + UVB group in SCC-25 and CAL-27 were 32.87% and 34.85%, respectively, compared to 14.85% and 13.62% in the CON group. Additionally, the G2/M phase proportions of the oeMIF + UVB group in SCC-25 and CAL-27 were 20.22% and 20.63%, and 14.38% and 15.14%, respectively, in the CON group (Figure 3B).

Annexin V PE and 7-AAD double staining was conducted for apoptosis detection in SCC-25 and CAL-27 cells after the corresponding treatments. As depicted in Figure 3C, the apoptotic rate of cells increased significantly 24 h post UVB

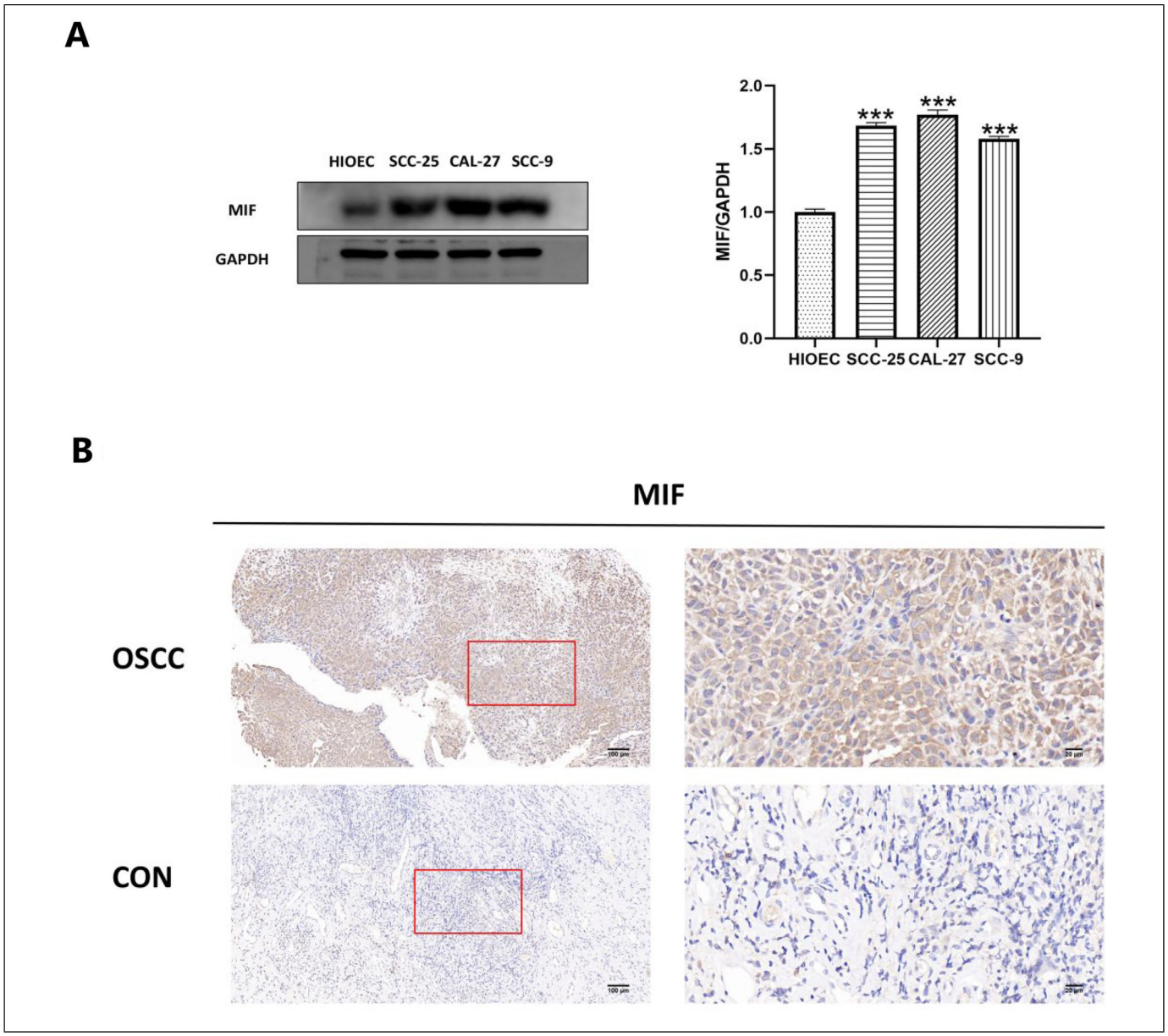

Figure 1. Expression of MIF in OSCC tissues and OSCC cell lines. (A) The expression of MIF in HIOEC, SCC-9, SCC-25 and CAL-27 was detected by western blotting. (B) Detection of MIF expression in OSCC and adjacent tissues by immunohistochemistry (×100 and ×400). Data are shown as mean ± SD values. CON: adjacent tissues; OSCC: OSCC tissues, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

irradiation. The apoptotic rates of the shMIF + UVB group in SCC-25 and CAL-27 cells were 42.9% and 57.35%, respectively, compared to 10.17% and 8.34%, respectively, in the CON group. Furthermore, the apoptosis rates of the oeMIF + UVB group in SCC-25 and CAL-27 cells were 20.59% and 36.14%, respectively, compared to 9.44% and 5.90%, respectively, in the CON group (Figure 3D). These data suggest that MIF knockdown combined with UVB irradiation promoted cell cycle arrest at the G2/M phase and prompted apoptosis in OSCC compared with the mono-treatment or non-treatment groups.

MIF Knockdown Combined With UVB Irradiation May Trigger Apoptosis in OSCC Cells Through the Mitochondrial Pathway

Mitochondrial homeostasis is essential for apoptosis regulation, and a drop in MMP is a characteristic feature of apoptosis.[19] Therefore, a JC-1 kit was used to measure changes in MMP expression in OSCC cells after MIF knockdown plus UVB irradiation. The results showed that the shMIF + UVB group had significantly decreased MMP in CAL-27 and SCC-25 cells compared to treated or untreated cells alone (Figure 4A).

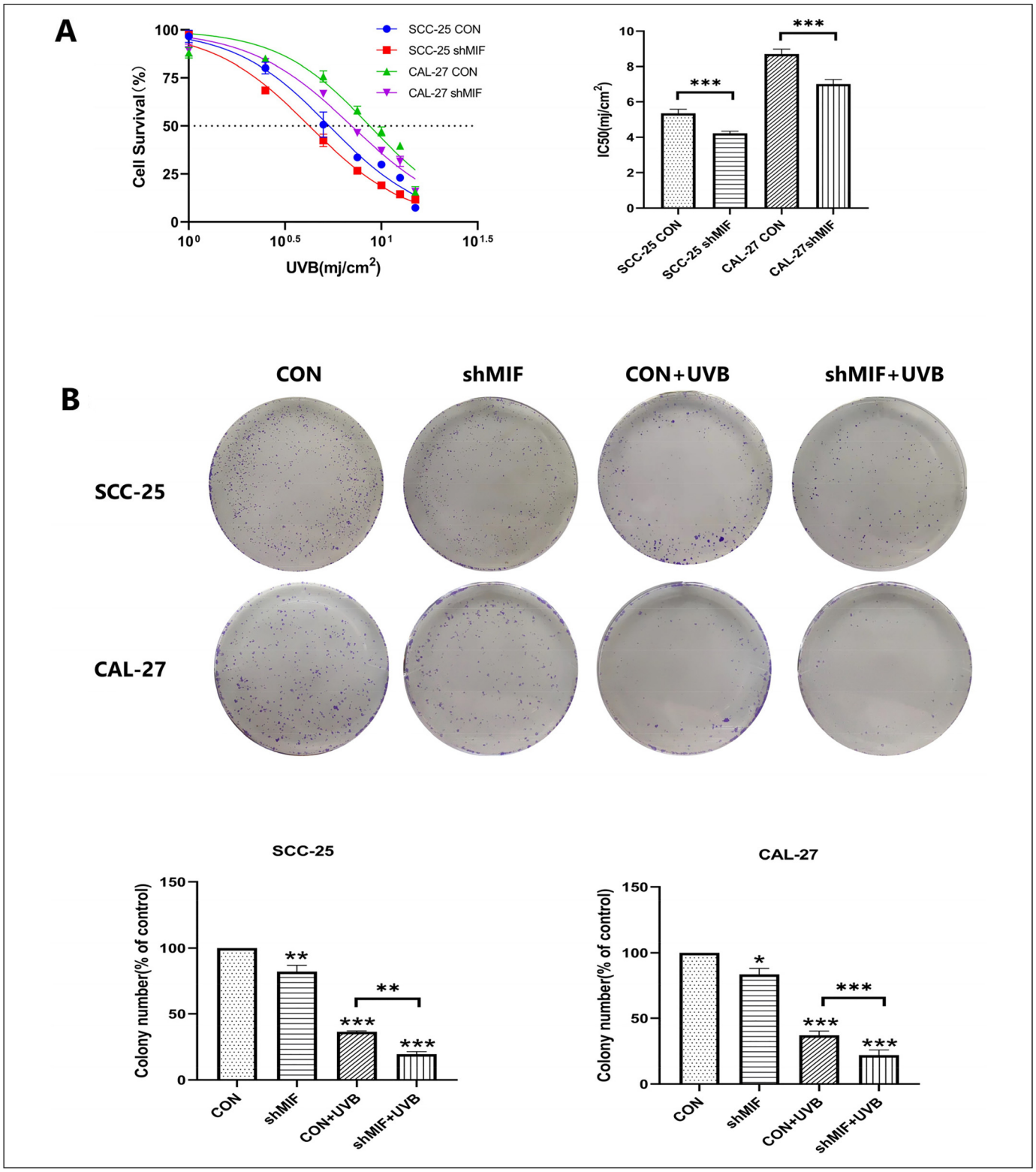

Figure 2. MIF knockdown combined with UVB irradiation inhibited OSCC cell viability and proliferation. (A) SCC-25 and CAL-27 cells in the CON and shMIF groups were treated with different doses of UVB irradiation (0, 1, 2.5, 5, 7.5, 10, 12.5, and 15 mJ/cm^2), and CCK-8 assay was performed for cell viability measuring. (B) The effect of UVB irradiation (5 mJ/cm^2) on the proliferation of SCC-25 and CAL-27 cells in the CON and shMIF groups.

Data are shown as mean ± SD values. *$P < .05$, **$P < .01$, ***$P < .001$.

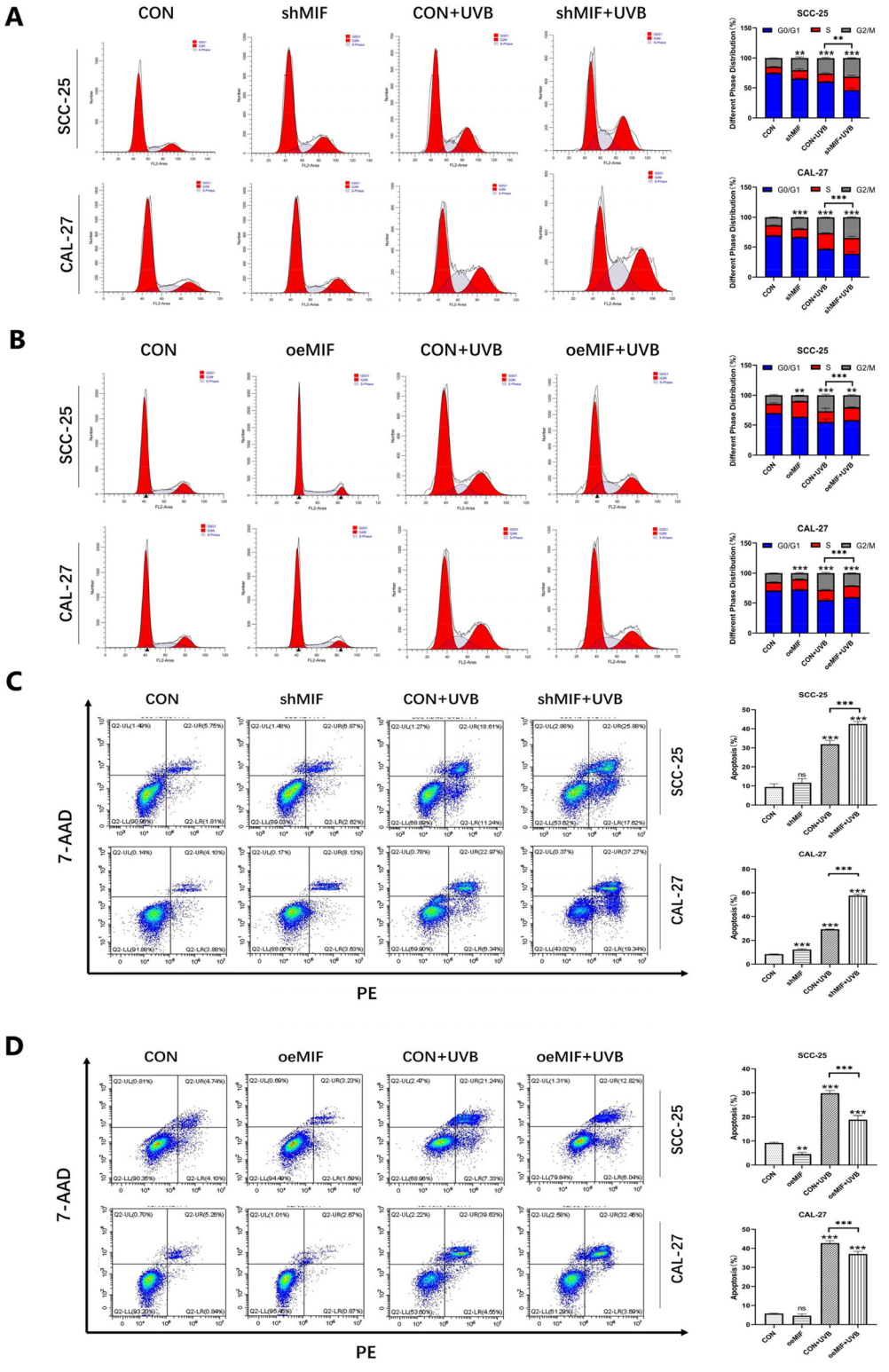

Figure 3. MIF knockdown or overexpression combined with UVB irradiation induced apoptosis in OSCC cells and altered the cell cycle distribution. In SCC-25 and CAL-27 cells, the CON and shMIF groups were treated with or without UVB irradiation before being collected after 24 h. (A and B) Propidium iodide (PI) staining was performed for cell cycle distribution assesssing via flow cytometry. (C and D) Apoptosis was assayed using Annexin V PE/7-AAD staining.
Data are shown as mean ± SD values. *$P < .05$, **$P < .01$, ***$P < .001$.

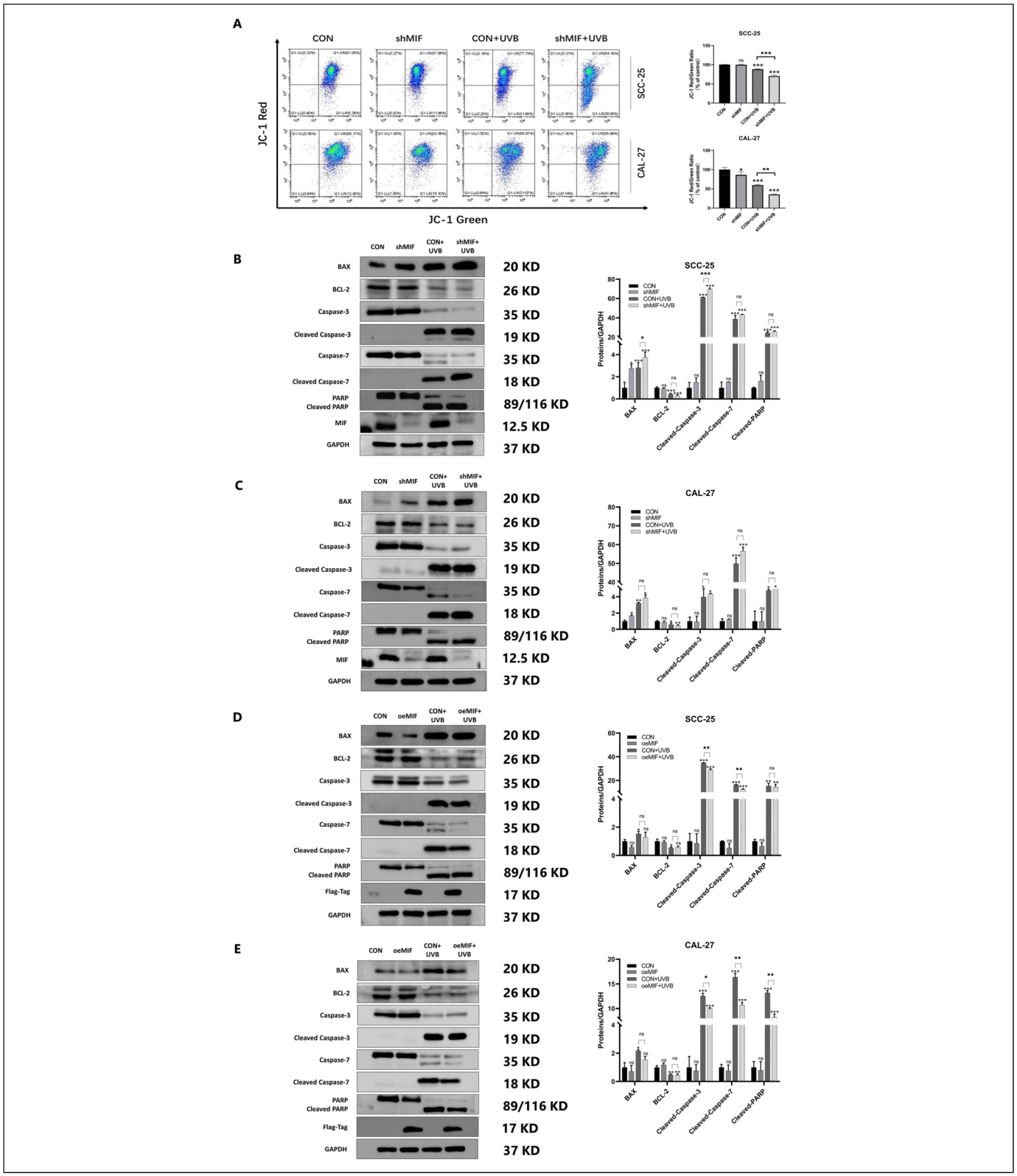

Figure 4. MIF knockdown combined with UVB irradiation triggers apoptosis via the mitochondrial pathway. (A) In CAL-27 cells, mitochondrial membrane potential was assayed with JC-1 staining in the CON and shMIF groups after treatment with or without UVB irradiation. (B and C) The effect of MIF knockdown and/or UVB irradiation on protein Bax, Bcl-2, caspase-3, Cleaved-caspase-3, caspase-7, Cleaved-caspase-7, and PARP in OSCC cells. (D and E) Effects of MIF overexpression and/or UVB irradiation on the expression of Bax, Bcl-2, caspase-3, Cleaved-caspase-3, caspase-7, Cleaved-caspase-7, and PARP in OSCC cells.
Data are shown as the mean ± SD values. * $P < .05$, ** $P < .01$, *** $P < .001$.

These results suggested that MIF knockdown combined with UVB irradiation induces mitochondrial depolarization, leading to apoptosis.

Bax and Bcl-2 both participate in mitochondria-mediated apoptosis. Bax activates caspases by permeabilizing mitochondrial membranes for cytochrome c release, acting as a pro-apoptosis protein, while Bcl-2 represses cytochrome c release, acting as an anti-apoptosis agent.[20] Thus, we detected the Bax and Bcl-2 expression in each group of cells using western blotting. The results depict a higher Bax expression in the shMIF + UVB group compared to CON, shMIF, and UVB groups of SCC-25 and CAL-27 cells. Meanwhile, Bcl-2 expression was significantly downregulated in the shMIF + UVB group compared to the CON, shMIF, and UVB groups in SCC-25 and CAL-27 cells (Figure 4B and C). Furthermore, compared to the UVB-only group, Bax expression was lowered while Bcl-2 expression was elevated in the oeMIF + UVB group (Figure 4D and E). These results suggest that MIF knockdown combined with UVB irradiation may promote apoptotic proteins but decrease anti-apoptotic protein levels in SCC-25 and CAL-27 cells.

Proteins from the caspase family play major roles in the apoptosis process, and caspase activation is a key event in this process.[21] Therefore, the expression of caspase-related proteins was studied. Western blotting demonstrated a reduction in caspase-3 and caspase-7 expression in the shMIF + UVB group compared to mono-treated or untreated SCC-25 and CAL-27 cells; however, cleaved-caspase-3 and cleaved-caspase-7 levels were significantly elevated. Besides, a significant reduction in intact PARP (116 kDa) and an increase in the cleaved form of PARP (89 kDa) were also observed (Figure 4B and C). Additionally, caspase-3 and caspase-7 expression was raised in the oeMIF + UVB group compared to the UVB group, with a decrease in cleaved-caspase-3 and cleaved-caspase-7 levels. However, the levels of intact PARP (116 kDa) were elevated, while those of the cleaved form PARP (89 kDa) were decreased (Figure 4D and E).

In conclusion, MIF knockdown combined with UVB irradiation could trigger apoptosis in OSCC via the mitochondrial pathway.

MIF Knockdown Combined With UVB Irradiation Induces Apoptosis via the MAPK Pathway

Previous studies have found that UVB irradiation activates the MAPK signaling pathway, ultimately leading to apoptosis.[22,23] Therefore, we detected the phosphorylated P38 and JNK 1/2 proteins in OSCC cells by Western blotting. Significantly higher phosphorylation levels of P38 and JNK 1/2 in shMIF + UVB groups were found compared to the mono-treated or untreated SCC-25 and CAL-27 cells (Figure 5A and B). Additionally, P38 and JNK 1/2 phosphorylation was weaker in the oeMIF + UVB group compared to the UVB group in both cell lines (Figure 5C and D).

ROS Generation as an Upstream Regulator in UVB Irradiation-Induced Apoptosis

ROS accumulation has been suggested to initiate apoptosis by triggering mitochondrial depolarization.[24] Changes in the ROS levels in each group of cells were assessed via flow cytometry analysis. As indicated in Figure 6A, MIF knockdown combined with UVB radiation brought a substantial leap in ROS levels. Additionally, MIF overexpression combined with UVB irradiation caused a decrease in ROS levels compared to cells irradiated with UVB alone (Figure 6B). However, MIF knockdown or overexpression alone did not significantly alter intracellular ROS levels. The results showed that MIF knockdown promoted intracellular ROS production following UVB irradiation. To further study the role of ROS in mediating UVB irradiation-induced apoptosis in CAL-27 cells, the cells were pretreated with 2 mM N-acetylcysteine (NAC), a ROS scavenger, for 1 h,[25] after which ROS levels and apoptosis were determined by flow cytometry. The results demonstrated that UVB irradiation-induced ROS generation was significantly inhibited by 2 mM NAC pretreatment (Figure 6C). Flow cytometry showed that combined treatment with NAC rescued MIF knockdown and UVB irradiation-induced apoptosis (Figure 6D). These results suggest that an enhancement of UVB-induced apoptosis could be achieved in CAL-27 cells by increased ROS accumulation.

The influence of NAC over MIF knockdown combined with UVB irradiation-induced MAPK activation was further evaluated. NAC pretreatment reversed MIF knockdown combined with UVB irradiation-induced P38 and JNK 1/2 phosphorylation, suggesting that ROS may mediate UVB irradiation-induced P38 and JNK 1/2 activation. Importantly, NAC blockade of ROS production resulted in a decrease in Bax and cleaved PARP expression and an increase in Bcl-2 expression (Figure 6E). These results suggest that ROS-mediated MIF knockdown activates the mitochondrial apoptotic pathway and may be a key upstream regulator of UVB irradiation-induced anticancer activity.

Discussion

The development and progression of malignant tumors are associated with many factors, among which reduced apoptosis and abnormal cell proliferation are key factors.[26] MIF is significantly overexpressed in various carcinomas, such as esophageal squamous cell[27] and nasopharyngeal carcinomas,[28] and cervical,[29] breast,[30] and colorectal cancers.[31] Some studies have also reported that MIF is overexpressed in OSCC and can promote OSCC proliferation, migration, and metastasis.[14,15] Similarly, our results showed that MIF expression was significantly elevated in all OSCC tissues and cell lines, suggesting that MIF is highly expressed in OSCC and may play a carcinogenic role, which echoes its pro-carcinogenic role in most cancers. UVB irradiation has been a popular treatment for various skin diseases owing to its advantage of low systemic toxic side effects. In this study, we found that in SCC-25 and

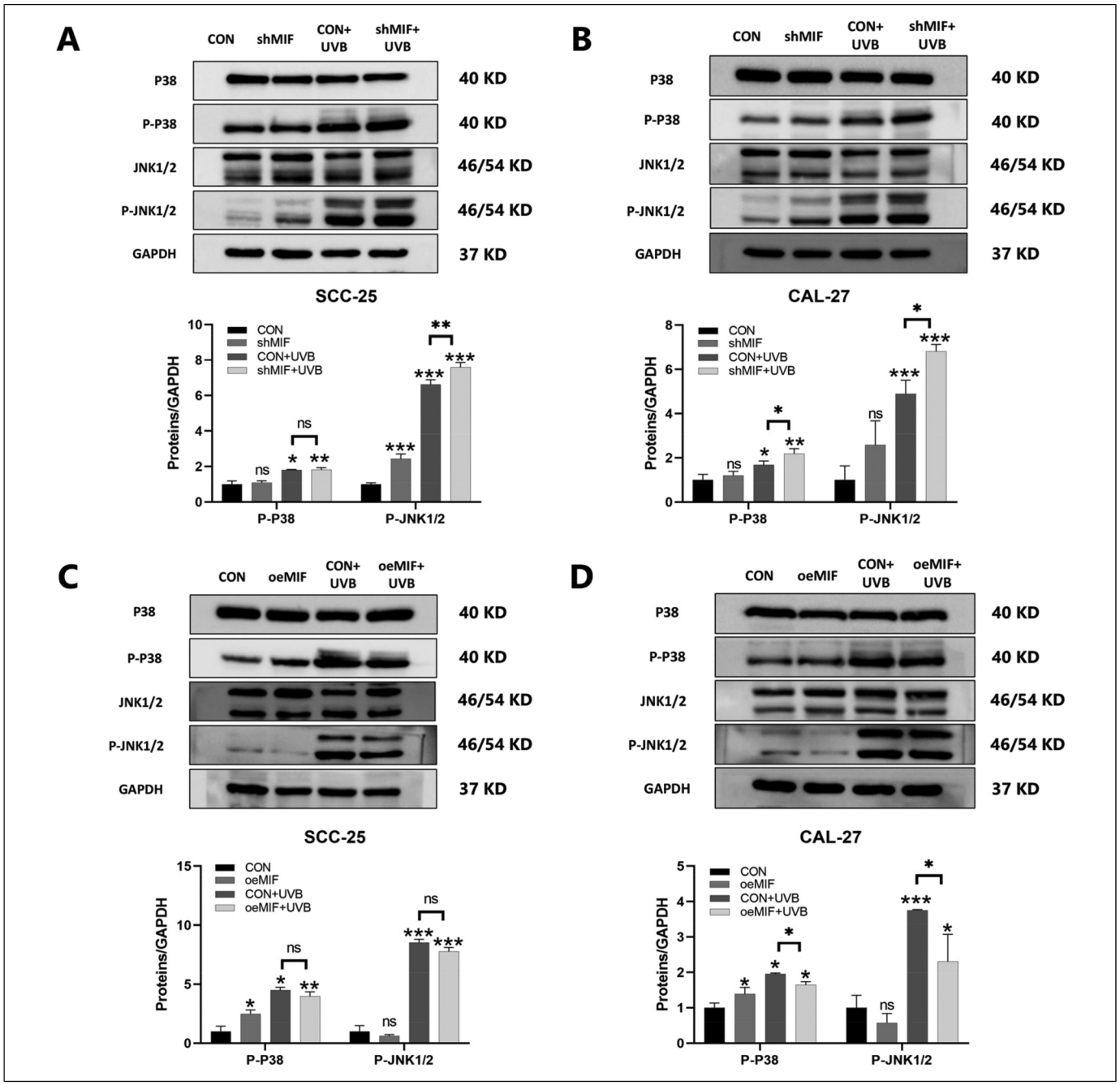

Figure 5. MIF knockdown combined with UVB irradiation triggers apoptosis via the MAPK pathway. (A and B) The effect of MIF knockdown and/or UVB irradiation on the expression of P38, P-P38, JNK 1/2, and P-JNK 1/2 in OSCC cells. (C and D) The effect of MIF overexpression and/or UVB irradiation on the expression of P38, P-P38, JNK1/2, and P-JNK1/2 in OSCC cells.
Data are shown as mean ± SD values. $*P < .05$, $**P < .01$, $***P < .001$.

CAL-27 cells, the IC_{50} value in the control group was significantly higher than that in the MIF-knockdown groups. This suggests that higher MIF levels in OSCC cells are more sensitive to UVB irradiation, whereas lower MIF levels in normal tongue epithelial cells help to avoid damage from UVB irradiation. This is noteworthy and highlights the potential of UVB irradiation in OSCC treatment. Therefore, we proposed a combination therapy using both MIF molecular targeting therapy and UVB irradiation and investigated the effects and potential mechanisms of this combination at the cellular level.

In this study, we found that MIF is a mediator of UVB-induced ROS generation; MIF knockdown combined with UVB irradiation may induce apoptosis through the ROS-dependent MAPK apoptosis pathway. The conclusion that MIF might exert influence on the process of UVB-inducing apoptosis in OSCC cells is supported by

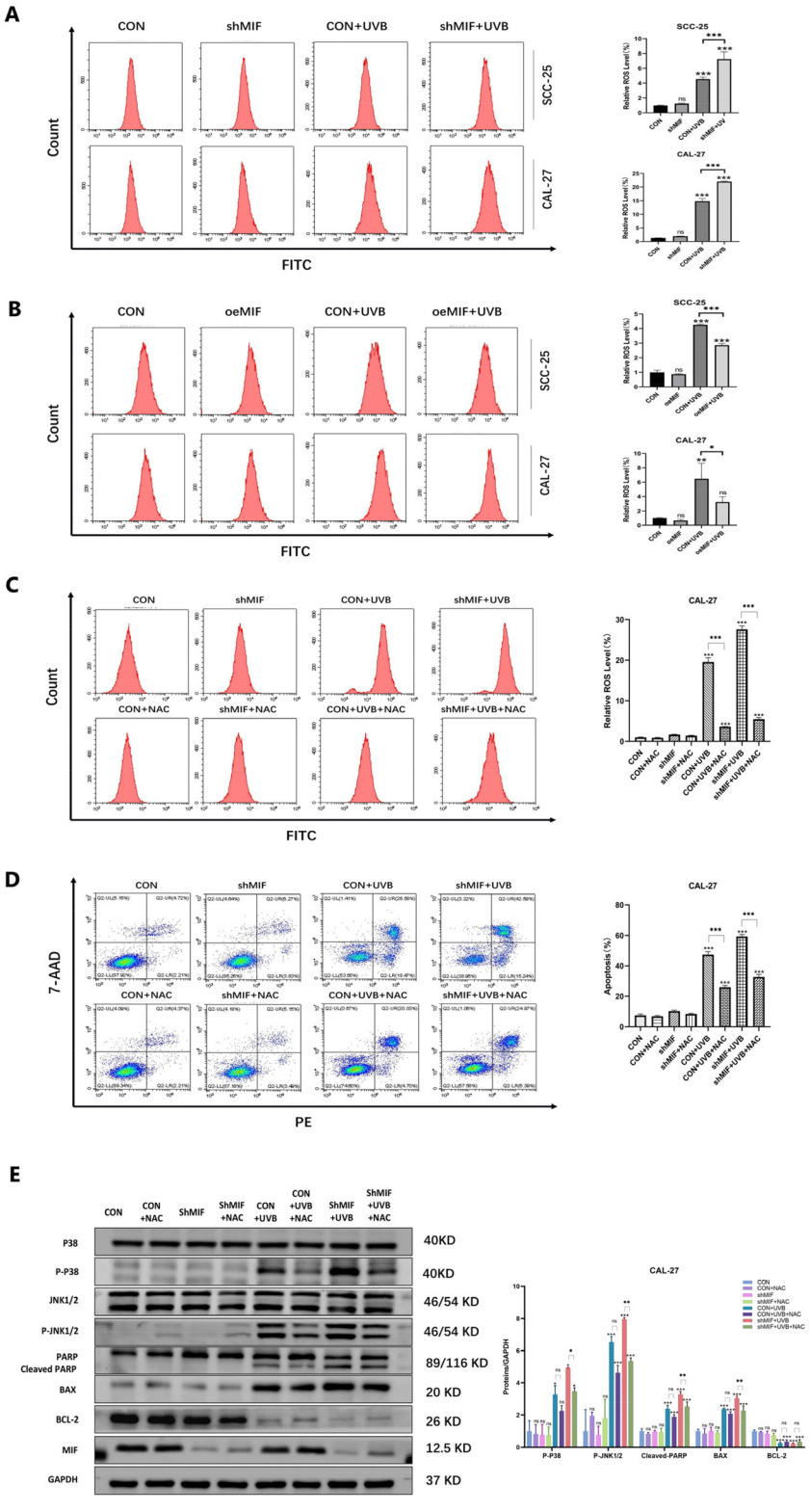

Figure 6. ROS generation appears as an upstream regulator in apoptosis induced by UVB irradiation. (A and B) ROS generation in OSCC cells treated with or without UVB irradiation was detected using flow cytometry. CAL-27 cells were incubated with or without 2 mM NAC for 1 h before being treated with or without UVB irradiation. The cells were collected after 24 h. (C) Intracellular ROS production was detected by flow cytometry. (D) Apoptosis detection was accomplished using an Annexin-V PE/7-AAD kit. (E) Protein PARP, P38, P-P38, Bax, and Bcl-2 were assayed by Western blotting.

Data are shown as mean ± SD values. $*P < .05$, $**P < .01$, $***P < .001$.

following outcomes: (1) MIF knockdown enhances sensitivity to UVB-induced apoptosis and increases apoptosis, and (2) MIF overexpression reduces UVB-induced apoptosis. Chuang et al found that MIF inhibition reduced intracellular ROS levels.[32] In this study, MIF knockdown alone slightly increased intracellular ROS levels, but the finding lacked statistical significance. Interestingly, the intracellular ROS content was significantly elevated in the shMIF + UVB group compared to the UVB group. This suggests the involvement of MIF in UVB-induced ROS generation; however, the mechanism remains unrevealed.

The effects of MIF knockdown and UVB irradiation in OSCC cells were also investigated. CCK-8 and clone formation assays were performed for cell proliferation detection. Results showed that UVB irradiation inhibited OSCC cell growth in a dose-dependent manner. Further, MIF knockdown combined with UVB irradiation had a better antiproliferative effect than that of MIF knockdown or UVB irradiation alone.

The apoptosis rate in the shMIF + UVB group was significantly higher than that in the CON, shMIF, and UVB groups, as confirmed by flow cytometry. The percentage of cells at the G2/M phase in the shMIF + UVB group was significantly higher than that in the CON, shMIF, and UVB groups. This suggested that MIF knockdown, UVB irradiation, or MIF knockdown combined with UVB irradiation inhibited cell proliferation and induced apoptosis, while MIF knockdown combined with UVB irradiation had the strongest anticancer effect.

UVB-induced apoptosis remains an unsolved and complicated phenomenon involving multiple signaling pathways, inducing DNA damage, cell death receptor activation, and ROS generation.[33] UVB is considered an effective intracellular ROS inducer,[34] and excessive ROS accumulation disrupts mitochondria, leading to the downregulation of MMP, which stimulates the release of cytochrome c in the cytoplasm and ultimately induces apoptosis.[35] Here, we found that ROS and MMP depolarization levels were significantly elevated in the shMIF + UVB group than in any other group. The oeMIF + UVB group had lower ROS levels than that of the UVB group. It could be concluded that MIF is involved in UVB-induced ROS generation and may play an important role in UVB-induced apoptosis in OSCC cells.

Mitochondria play a pivotal role in the apoptosis process.[36] As a regulator in the mitochondrial apoptotic pathway, Bcl-2 family proteins regulate cytochrome c release by altering mitochondrial outer membrane permeability and cytochrome c, caspase-9, caspase-3, and PARP cleavage to activate the mitochondria-mediated apoptotic pathway.[37] Western blotting showed that the shMIF + UVB group had elevated Bax expression and reduced Bcl-2 expression compared to the CON, shMIF, and UVB groups. Additionally, cleaved-caspase-3, cleaved-caspase-7, and cleaved-PARP levels were also significantly increased. However, compared to the mono-treatment UVB group, the oeMIF + UVB group demonstrated lower levels of Bax, cleaved-caspase-3, cleaved-caspase-7, and cleaved-PARP and higher levels of Bcl-2. Thus, the cytotoxic effect of MIF knockdown combined with UVB irradiation on OSCC cells may be caused by mitochondrial apoptosis pathway activation.

The MAPK signaling pathway plays a key role in cell growth, migration, proliferation, differentiation, and apoptosis, and its family members mainly include extracellular signal-regulated kinase (ERK), stress-activated protein kinase (JNK), and P38 mitogen-activated protein kinase (P38MAPK). Wang et al showed that MIF is significantly upregulated in osteosarcoma and promotes cancer cell proliferation and migration through the activation of the MAPK signaling pathway.[38] Jiang et al found that Kin17 promotes thyroid cancer cell proliferation, migration, and invasion by activating the P38 MAPK signaling pathway in vivo.[39] P38 MAPK and JNK 1/2, as members of the MAPK family, play important roles in the apoptotic signaling pathway. Nys, K et al showed that UVB radiation induces apoptosis in human keratinocytes through activation of P38 MAPK.[40] Moriyama, M et al found that UVB activation of JNK 1/2 and ERK1/2 MAPK in human primary epidermal keratinocytes induces apoptosis.[41] Several studies have shown that ROS-induced apoptosis can be mediated by P38 and JNK 1/2 activation.[42,43] In our study, the shMIF + UVB group showed significantly increased P38 and JNK 1/2 phosphorylation levels compared to either treatment alone. The application of the ROS inhibitor NAC inhibited UVB-induced P38 and JNK 1/2 phosphorylation. Thus, we concluded that MIF mediates UVB-related ROS accumulation and may enhance UVB irradiation-induced apoptosis via the MAPK pathway.

Overall, this study demonstrated that MIF knockdown combined with UVB inhibited OSCC cell proliferation and promoted the apoptosis of OSCC cells at the cellular level. However, this study had some limitations. First, the sample size of OSCC patients in this study was small and there may be errors. Additionally, the corresponding receptors in MIF knockdown or overexpressed OSCC cells were not determined. The specific mechanism of MIF involved in UVB-induced ROS generation and the effects of MIF knockdown combined with UVB on OSCC cell proliferation and apoptosis in vivo still warrant further study.

Conclusion

MIF knockdown is a regulator in UVB-induced ROS generation, which enhances the effect of UVB on OSCC cells by inhibiting tumor cell proliferation and activating the MAPK pathway to promote apoptosis. Collectively, our findings suggest that MIF knockdown combined with UVB irradiation is a prospective approach for OSCC treatment. Therefore, MIF-targeted inhibitors combined with UVB irradiation may be a new therapeutic approach to reduce the complications of conventional treatments for OSCC.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics Statement

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