Article

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Curcumin Mediates Macrophage Polarization by Regulating the AXIN2/β-catenin Pathway, Inhibits Inflammatory Responses and Hinders the Progression of Oral Squamous Cell Carcinoma

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Background: Macrophage polarization to M2 type plays a key role in promoting the progression of oral squamous cell carcinoma (OSCC). Curcumin, as a natural active ingredient, has been proven to have anti-tumor activity, but its regulatory mechanism on macrophage polarization in OSCC remains unclear. This study aims to explore whether curcumin affects the M2 polarization of macrophages by regulating the axis inhibition protein 2 (AXIN2)/β-catenin pathway, and to clarify its effects and molecular mechanisms on the growth and invasion of OSCC cells.

Methods: RAW264.7 cells were selected to be treated with different concentrations (0, 5, 10, 20 µM) of curcumin, and the expression of M2 markers was detected by Real-time fluorescence quantitative PCR (qRT-PCR) and flow cytometry. The expression of AXIN2/β-catenin pathway molecules and downstream genes was detected using Western blotting. The localization and expression intensity of arginase-1 (ARG1) and β-catenin were observed using immunofluorescence staining. The secretion levels of cytokines were detected by enzyme-linked immunosorbent assay (ELISA). The conditioned medium of curcumin-pre-treated macrophages was collected and used to treat OSCC cell lines (HSC3, CAL33). Cell viability, proliferation, apoptosis and invasion ability were evaluated. The role of AXIN2 in curcumin-regulated macrophage polarization and OSCC cell behavior was verified by knockdown of AXIN2 in macrophages.

Results: Curcumin inhibited the polarization of M2 type macrophages in a concentration-dependent manner ($p < 0.01$). Mechanistically, curcumin upregulated AXIN2 expression in macrophages and inhibited the nuclear translocation of β-catenin and the expression of its downstream target genes ($p < 0.01$). The conditioned medium from curcumin-pre-treated macrophages significantly inhibited the viability, colony formation ability, and invasion ability of OSCC cells, and promoted cell apoptosis ($p < 0.01$). Knockdown of AXIN2 partially reversed the inhibitory effect of curcumin on M2 polarization of macrophages and weakened the inhibitory effect of the conditioned medium on the growth and invasion of OSCC cells ($p < 0.01$).

Conclusions: Curcumin inhibits the activation of the β-catenin pathway by up-regulating AXIN2 expression, thereby blocking the polarization of macrophages to the M2 type, reducing the secretion of pro-cancer cytokines, and ultimately suppressing the growth and invasion of OSCC cells. This study reveals a new mechanism by which curcumin regulates the polarization of macrophages in the tumor microenvironment, providing experimental evidence for targeted therapy of OSCC.

Keywords: curcumin; macrophage; AXIN2/β-catenin; oral squamous cell carcinoma

Introduction

Oral squamous cell carcinoma (OSCC) ranks among the most prevalent malignant neoplasms in the oral and maxillofacial area, accounting for approximately 90%. It is commonly caused by smoking, drinking alcohol, and viral infections, and seriously affects the health of patients and imposes a heavy social and economic burden [1,2]. Although certain specific progress has been made regarding the diagnosis and treatment of OSCC, its 5-year survival rate remains below 60%, and the prognosis is not optimistic [3]. OSCC is characterized by highly invasive and metastatic, easily invading surrounding tissues and distant organs, which leads to a significant decline in the quality of life of patients [2]. Presently, the main treatment methods for OSCC include surgical resection, radiotherapy, and chemotherapy, but all these methods have certain limitations [4]. Therefore, it is of great urgency to find an efficient and low-toxic treatment method or an auxiliary treatment approach.

Growing evidence from studies indicates that the tumor microenvironment (TME) is crucial in the occurrence,

development, and metastasis of OSCC. Macrophages, as an important component of the tumor microenvironment, exhibit pronounced plasticity and heterogeneity, and can polarize into M1 and M2 types of macrophages based on the signals from the microenvironment. M1-type macrophages have strong pro-inflammatory and anti-tumor activities, while M2-type macrophages exhibit anti-inflammatory and pro-tumor characteristics, secreting immunosuppressive factors such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), which promote the growth and survival of tumor cells and inhibit the anti-tumor immune response of the body [5,6]. In OSCC TME, the proportion of M2 type macrophages tends to increase, which is associated with the malignancy of the tumor and the poor prognosis [7]. Research has revealed that promoting the polarization of M2-type macrophages and creating a chronic inflammatory microenvironment can facilitate the progression of OSCC [8]. Therefore, regulating the polarization direction of macrophages and inhibiting their transformation into M2-type macrophages is expected to become a new strategy for treating OSCC.

The axis inhibition protein 2 (AXIN2)/β-catenin pathway constitutes a key component of the Wnt signaling pathway and plays an essential role in diverse physiological processes, such as cell proliferation, differentiation, migration, and chronic inflammation. Abnormal activation of this pathway is associated with the development of various tumors [9]. When the classical Wnt signaling pathway is not activated, AXIN2 forms a degradation complex. β-catenin is phosphorylated and then ubiquitinated and degraded through the proteasome pathway, maintaining a low level of β-catenin in the cell. At this time, β-catenin cannot enter the cell nucleus and thus fails to activate downstream target genes [10]. During the process of tumor occurrence and development, the AXIN2/β-catenin-related pathway often becomes dysregulated. In colorectal cancer (CRC), activating AXIN2 and inhibiting the Wnt/β-catenin pathway can thereby suppress the growth of tumors [11]. In OSCC, the dysregulation of the Wnt/β-catenin pathway promotes OSCC progression and metastasis, with such abnormal pathway activation being triggered by β-catenin's irregular expression [12]. The AXIN2/β-catenin signaling pathway, when down-regulated, can inhibit the proliferation and migration of tumor cells in OSCC. The β-catenin inhibitor can partially reverse its related carcinogenic effects [13]. Therefore, the AXIN2/β-catenin pathway has become an important potential target for cancer treatment.

Curcumin is a natural polyphenolic compound extracted from the roots and stems of the plant Curcuma longa in the ginger family. It displays a broad spectrum of biological activities, including antioxidant and anti-inflammatory properties, antibacterial, and anti-tumor effects [14–16]. A large body of research has indicated that curcumin can inhibit the proliferation of tumor cells, induce cell apoptosis, inhibit tumor angiogenesis and metastasis through multiple pathways, and has shown potential application value in the prevention and treatment of various cancers [17]. For instance, curcumin activates nuclear factor erythroid 2-related factor 2 (NRF2) through reactive oxygen radicals (ROS), induces miR-34a, promotes mesenchymal-epithelial transition, and inhibits the metastasis of CRC in mice [18]. Moreover, curcumin can inhibit the epithelial-mesenchymal transition (EMT) of OSCC cells induced by hepatocyte growth factor (HGF) by blocking c-Met and inhibiting the extracellular signal-regulated protein kinases (ERK) pathway, and can also reduce cell motility by inhibiting the gelatin dissolution activity induced by HGF [19]. Curcumin might hinder the proliferation of OSCC cells via a mechanism dependent on specificity protein 1 (Sp1)/nuclear factor kappa-B (NF-κB) [20]. However, the role and mechanisms of curcumin in the treatment of OSCC remain unclear, especially regarding its regulation of macrophage polarization and inflammatory responses. Therefore, in-depth exploration of the effects of curcumin on OSCC and its underlying mechanisms not only helps to reveal the pathogenesis of OSCC and provide new theoretical basis for its treatment, but also holds the potential to lay the foundation for the development of novel, highly effective, and low-toxicity therapeutic drugs for OSCC.

Materials and Methods

Cell Culture and Processing

RAW264.7 (10350) murine mononuclear macrophage leukemia cells, HSC3 (17062) and CAL33 (16776) OSCC cell lines were all purchased from Wanwu Biological Co., Ltd. (Hefei, China). The cells were cultured in DMEM medium (11995065, Gibco, NY, USA) containing fetal bovine serum (FBS, 164210-50, Procell, Wuhan, China) and 1% penicillin/streptomycin (PB180120, Procell, Wuhan, China), and maintained in a 37 °C, 5% CO_2 humidified incubator. Curcumin (HY-N005) was purchased from MedChemExpress (NJ, USA). RAW264.7 cells were induced to M2 type using IL-4 (20 ng/mL) (P00196, Solarbio, Beijing, China). According to previous literature reports, when the concentration of curcumin exceeded 40 µM, the viability of RAW264.7 cells significantly decreased; concentrations (0, 5, 10, 20 µM) that were non-toxic to macrophages were selected for the experiments [21]. Next, the cells were cultured in the incubator for 24 hours. After centrifugation at 12,000 rpm for 10 minutes, the supernatant was passed through a 0.45 µm filter membrane to obtain the conditioned medium. All cells have undergone STR analysis and mycoplasma testing, confirming that the cell identity was correct and absence of contamination.

Flow Cytometry

Macrophages treated with curcumin for 24 hours were collected. Fluorescently labeled CD206-APC (F2120603)

antibodies obtained from MULTI SCIENCES (Hangzhou, China) were added, respectively, and incubated in the dark at 4 °C for 30 minutes. After washing three times with PBS, the cells were analyzed using flow cytometer (CytoFLEX, BECKMAN, USA). The proportion of $CD206^+$ cells among the total cells was analyzed using FlowJo 10.0 software (BD Biosciences, San Jose, CA, USA). During flow cytometry data processing, the detection index of the curcumin group was set as the benchmark.

Cell Transfection

Macrophages were seeded in a 6-well plate and cultured until the confluence reached 50% to 60%, at which point transfection was performed. According to the instructions of Lipofectamine 3000 (L3000008, Thermo Fisher, California, USA), the AXIN2 siRNA (5′-GGGUUUAUGUAUAAAUAUAUU-3′) or negative control siRNA (5′-UUCUCCGAACGUGUCACGUTT-3′) was mixed with the transfection reagent, and subsequently added to the cells. After transfection, cells were cultured for 48 hours. The knockdown efficiency of AXIN2 was verified using Western blotting. The transfected macrophages were treated with 20 μM curcumin for 24 hours, and the cells and conditioned media were collected for subsequent polarization detection and OSCC cell function experiments.

Real-time Fluorescence Quantitative PCR (qRT-PCR)

The processed macrophages were lysed using TRIzol reagent (15596026, Thermo Fisher, California, USA) for total RNA extraction. Subsequently, the isolated RNA was reverse-transcribed into cDNA using PrimeScript RT reagent Kit (RR037A, Takara, Tokyo, Japan). qRT-PCR was then performed using SYBR Green PCR Master Mix (RR420A, Takara, Tokyo, Japan) with the cDNA as template. The primer sequences were as follows: Arginase-1 (ARG1) forward 5′-AGCACTGAGGAAAGCTGGTC-3′, reverse 5′-TACGTCTCGCAAGCCAATGT-3′; CD206 forward 5′-ATTCCGGTCGCTGTTCAACT-3′, reverse 5′-AACGGAGATGGCGCTTAGAG-3′; CD163 forward 5′-TGGGATCGCCGTGACGCTTC-3′, reverse 5′-CAGCGACTGCCTCCACCGAC-3′; GAPDH forward 5′-TGTCTCCTGCGACTTCAACA-3′, reverse 5′-GGTGGTCCAGGGTTTCTTACT-3′. The $2^{-\Delta\Delta Ct}$ method was employed to calculate the relative expression levels of the genes, with GAPDH serving as an internal reference gene.

Enzyme-linked Immunosorbent Assay (ELISA)

The supernatant of macrophage culture was collected and centrifuged. The resulting supernatant was subjected to cytokine measurement using ELISA kits (JYMBIO, Wuhan, China) for IL-10 (JYM0005Mo) and TGF-β (JYM0144Mo) according to the manufacturer's instructions. Briefly, the assay was performed as follows: The enzyme-linked plate was set with 10 standard sample wells. Blank wells and test wells (add 10 μL of test sample + 40 μL of diluent) were set up. Except for the blank wells, 50 μL of enzyme-labeled reagent was added to each well. The dilution washing solution was washed 5 times and dried. Subsequently, 50 μL of chromogenic agent A and B were added, respectively. The reaction was stopped with stop solution, and the OD value at 450 nm was measured within 15 minutes, with the blank wells set as zero. The concentration of cytokines in the samples was calculated based on the standard curve.

Immunofluorescence Staining

Macrophages were inoculated onto 24-well plates precoated with slides. After treatment with curcumin for 24 hours, the cells were fixed with 4% paraformaldehyde (P0099, Beyotime, Shanghai, China), permeabilized with 0.3% Triton X-100 (T8200, Solarbio, Beijing, China) for 10 minutes, and then blocked with 5% BSA (SL038, Solarbio, Beijing, China) at room temperature for 30 minutes. The ARG1 or β-catenin antibody (16001-1-AP or 51067-2-AP, Proteintech, Wuhan, China) was added and incubated. The samples were washed three times with PBST, and then 1:500 Cy3-labeled goat anti-rabbit secondary antibody (K1034G, Solarbio, Beijing, China) was added for incubation in the dark. The cells were stained with DAPI (C0060, Solarbio, Beijing, China) for 5 minutes to visualize the nuclei, and then sealed with an anti-fluorescence quenching mounting agent. Images were observed and captured using a fluorescence microscope (CKX53, OLYMPUS, Tokyo, Japan) and the ARG1 fluorescence intensity and the nuclear localization ratio of β-catenin were analyzed.

Cell Counting Kit-8 (CCK-8)

OSCC cells (HSC3, CAL33) were seeded in a 96-well plate. Then, the supernatant of each group of macrophages was added and the cells were cultured for 24, 48, and 72 hours. 10 μL of CCK-8 reagent (MA02128-5, Meilunbio, Dalian, China) was added and incubated at 37 °C for 2 hours. A microplate reader (DR-200Bs, Diatek, Wuxi, China) was used to measure the absorbance at 450 nm, followed by the calculation of cell viability.

Clonogenic Assay

OSCC cells were seeded in a 6-well plate. The supernatant of each group of macrophages was added. The culture medium was refreshed every 3 days and continued for 14 days. Afterward, the cells were fixed using 4% paraformaldehyde, followed by staining with 0.1% crystal violet (G1064, Solarbio, Beijing, China), washed with PBS, dried and finally visualized under a microscope (XD-202, Sunny Optical, Nanjing, China), and the number of clonogenic cells was calculated.

Terminal Deoxynucleotidyl Transferase mediated dUTP Nick-End Labeling (TUNEL)

Apoptosis was detected using a TUNEL kit (K1133, APExBIO, Houston, USA) following the manufacturer's instructions. Follow the instructions of the TUNEL kit (K1133, APExBIO, Houston, USA). Briefly, cells were fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton X-100. The TUNEL reaction solution was then applied and the samples were incubated at 37 °C in the dark. Nuclei were stained with DAPI. Apoptotic cells were observed under a fluorescence microscope, and the apoptosis rate was calculated as the number of apoptotic cells / total cell number × 100%.

Transwell

Matrigel matrix gel was diluted with medium, 50 µL was placed in each well of the upper chamber of Transwell, and incubated at 37 °C to allow it to solidify. 200 µL of the cell suspension was added to the upper chamber, and the supernatants of each group of macrophages were added to the lower chamber. The samples were incubated at 37 °C for 24 hours. After fixation and staining with crystal violet, non-invasive cells in the upper chamber were gently removed using a cotton swab. The number of cells that had migrated through the membrane was then counted under a microscope.

Western Blotting

The treated macrophages were collected and processed, and RIPA lysis buffer (20101, YEASEN, Shanghai, China) was lysed on ice, then centrifuged for the supernatant. Nuclear protein was extracted using the nuclear cytoplasm separation kit (P0028, Beyotime, Shanghai, China) following the manufacturer's instructions. Protein concentration was determined by BCA method (E-BC-K318-M, Ebscience, Wuhan, China), and 30 µg of protein was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after adjusting the concentration. Following protein transfer onto a PVDF membrane via the wet transfer method, the membrane was blocked with 5% skimmed milk at room temperature for 1 hour. The primary antibodies from Proteintech (Wuhan, China) with dilution of 1:1000: AXIN2 (20540-1-AP), β-catenin (51067-2-AP), Cyclin D1 (60186-1-Ig), c-Myc (67447-1-Ig), GAPDH (60004-1-Ig), and Histone H3 (17168-1-AP) were added and incubated at 4 °C overnight; after washing three times with TBST, the membrane was incubated with the secondary antibodies (ZB-2305 and ZB-2301, ZSBIO, Beijing, China) at a dilution of 1:2000 for 1 hour. The image was collected by Tanon-4600 imaging system (Tanon, Shanghai, China), and the gray value of the bands was analyzed using ImageJ 1.41 software (National Institutes of Health, Bethesda, USA), expressed as the gray ratio of the target protein to the internal reference protein relative expression level.

Statistical Analysis

All experimental data were reported as mean ± standard deviation ($\bar{x} \pm s$), with GraphPad Prism 9.0 software (Graphpad Software, San Diego, CA, USA) used for statistical analysis. One-way analysis of variance (ANOVA) was adopted for multi-group comparisons, and the independent sample *t*-test was chosen for two-group comparisons. Statistical significance was determined when the p value was <0.05.

Results

Curcumin Inhibits the Polarization of Macrophages to the M2 Type and Reduces the Secretion of Pro-inflammatory Factors

To investigate the impact of curcumin on macrophage polarization, we first treated macrophages with varying concentrations of curcumin (0, 5, 10, and 20 µM) for 24 hours. qRT-PCR was used to assess the expression of surface markers of M2 macrophages. The results demonstrated that curcumin treatment led to a concentration-dependent reduction in the expression of Arg1, CD163 and CD206 compared to the control group (0 µM). Notably, the 20 µM curcumin group exhibited the most significant decrease ($p < 0.01$) [Fig. 1a–c]. Immunofluorescence staining further confirmed this result by demonstrating a marked decrease in the fluorescence intensity of ARG1 in curcumin-treated macrophages [Fig. 1d,e], providing direct evidence of its inhibitory effect on M2 polarization ($p < 0.001$). To validate the effect of curcumin on macrophage secretory function, we collected supernatants from each treatment group and measured IL-10 and TGF-β levels using ELISA. Curcumin treatment significantly reduced the secretion of both cytokines ($p < 0.01$) [Fig. 1f,g]. These findings indicate that curcumin primarily inhibits the macrophage M2 polarization and secretion of M2 macrophage-associated cytokines.

Conditioned Medium From Curcumin-treated Macrophages Suppresses the Growth of OSCC Cells

To evaluate the impact of curcumin-treated macrophages on OSCC cell growth, we collected conditioned media from macrophages treated with curcumin or vehicle and used them to culture HSC3 and CAL33 cells. Since the concentration of curcumin at 20 µM exhibited the most significant inhibitory effect on the polarization of macrophages to the M2 phenotype, a curcumin concentration of 20 µM was chosen for the subsequent experiments. CCK-8 assays revealed that curcumin-treated conditioned medium significantly reduced the viability of HSC3 and CAL33 cells ($p < 0.001$) [Fig. 2a,b]. Colony formation assays further confirmed that the curcumin treatment group exhibited fewer colonies than the control group in HSC3 and CAL33 cells ($p < 0.001$) [Fig. 2c,d], indicating that curcumin-pretreated macrophages impair

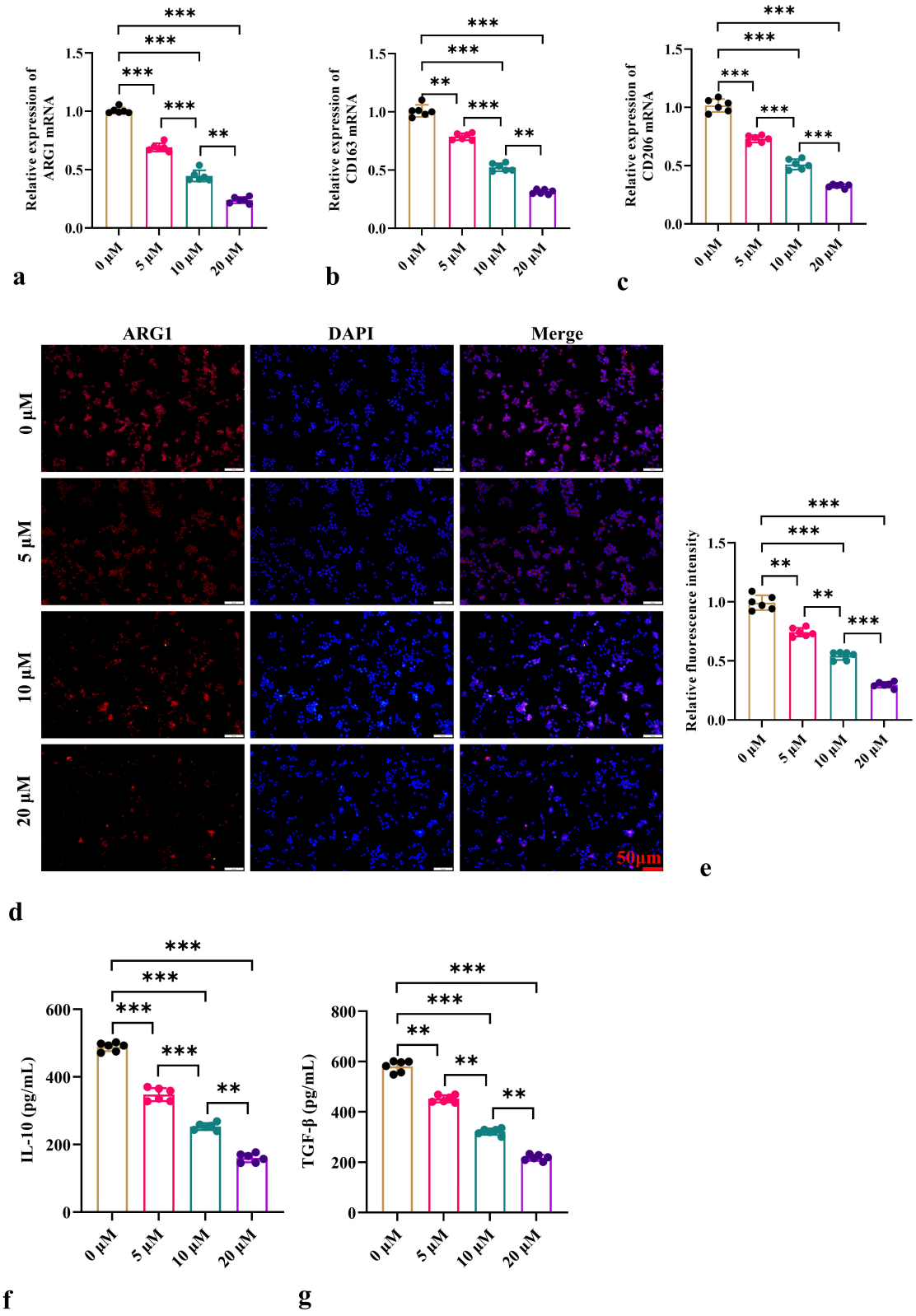

Fig. 1. Curcumin inhibits the polarization of macrophages to the M2 type and reduces the secretion of pro-inflammatory factors. (a–c) Expression of macrophage M2 polarization marker arginase-1 (ARG1), CD163 and CD206 was determined by Real-time fluorescence quantitative PCR (qRT-PCR) after treatment with different concentrations of curcumin. (d,e) The M2 polarization marker ARG1 in macrophages in the different concentrations of curcumin treatment groups was detected using immunofluorescence analysis. (f,g) The levels of interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) in the different concentrations of curcumin treatment groups was quantified using enzyme-linked immunosorbent assay (ELISA). n = 6; $^{**}p < 0.01$, $^{***}p < 0.001$.

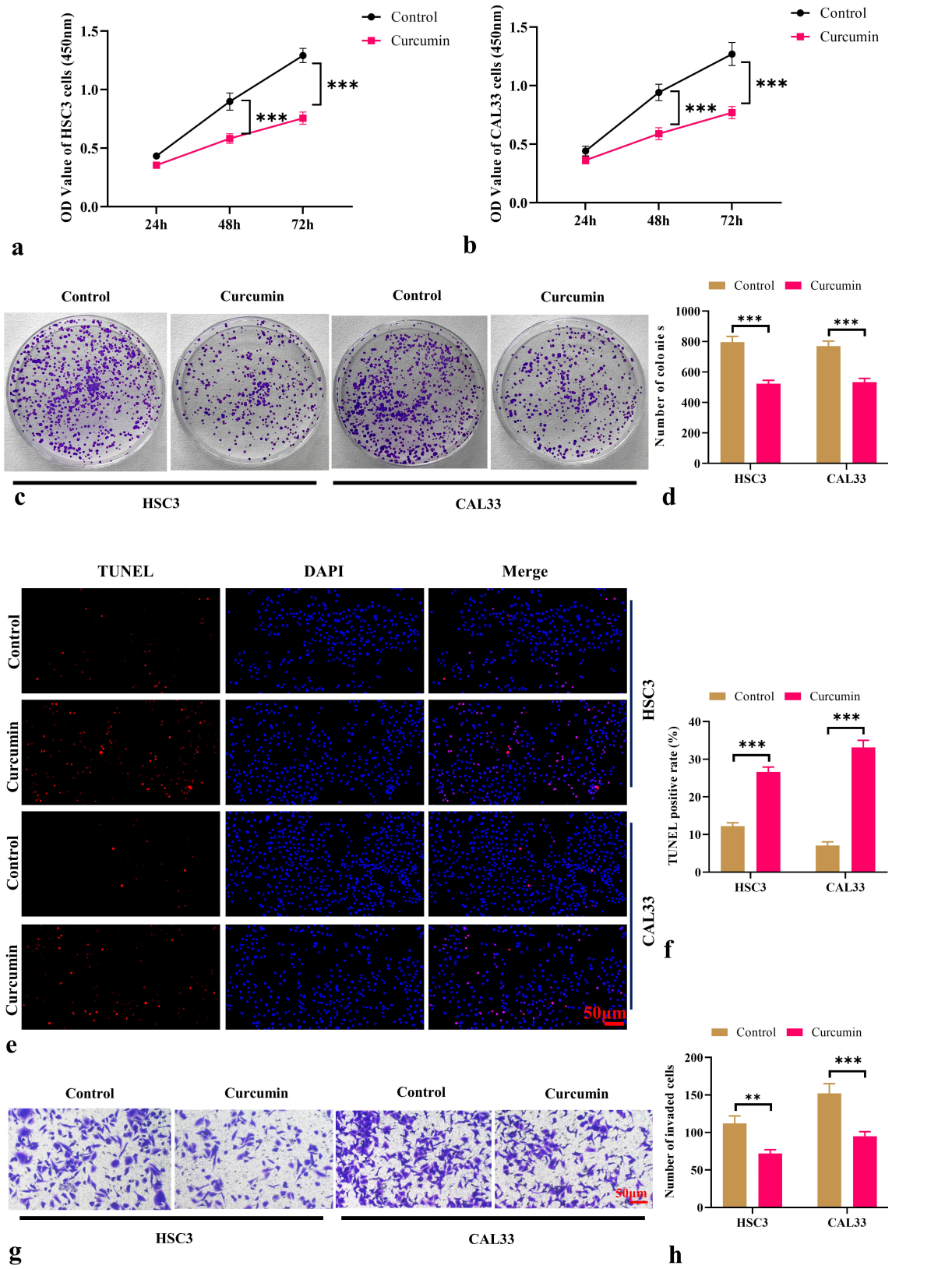

Fig. 2. Conditioned medium from curcumin-treated macrophages suppresses the growth of OSCC cells. (a,b) The cell viability was measured using Cell Counting Kit-8 (CCK-8) assay after treatment with 20 μM curcumin. (c,d) The proliferative capacity of cells was determined using clonogenic assay after treatment with 20 μM curcumin. (e,f) The apoptotic cells were identified using TUNEL assay after treatment with 20 μM curcumin. (g,h) The cell invasion potential was evaluated using Transwell assay after treatment with 20 μM curcumin. n = 6; $^{**}p < 0.01$, $^{***}p < 0.001$.

the clonogenic capacity of OSCC cells. To explore whether the growth inhibition was linked to apoptosis, TUNEL assays were performed. A notable increase was observed in apoptosis rates of HSC3 and CAL33 cells treated with curcumin-conditioned medium ($p < 0.001$) [Fig. 2e,f], suggesting apoptosis contributes to reduced cell viability. Additionally, Transwell invasion assays demonstrated a marked decrease in the number of OSCC cells penetrating the Matrigel barrier after curcumin treatment, indicating that curcumin also inhibits OSCC cell invasiveness ($p < 0.01$) [Fig. 2g,h].

Curcumin Upregulates AXIN2 Expression and Suppresses β-catenin Pathway Activation in Macrophages

To unravel the molecular mechanism by which curcumin regulates macrophage polarization, we examined the expression and localization of key molecules in the AXIN2/β-catenin pathway. From Fig. 3a,b, curcumin treatment significantly increased AXIN2 protein levels compared to the control group, while concurrently reducing translocation to the cell nucleus of β-catenin ($p < 0.01$). Immunofluorescence experiments further validated these changes: in control cells, β-catenin was predominantly localized in the nucleus with strong fluorescence, whereas curcumin-treated cells showed weakened nuclear signals ($p < 0.001$) [Fig. 3c,d]. Moreover, the protein levels of Cyclin D1 and c-Myc were markedly downregulated, confirming that curcumin represses β-catenin transcriptional activity ($p < 0.001$) [Fig. 3e,f].

AXIN2/β-catenin Pathway is a Critical Mediator of Curcumin-regulated Macrophage Polarization

To verify the necessity of AXIN2 in curcumin-mediated inhibition of M2 polarization, we knocked down AXIN2 in macrophages. After knockdown of AXIN2, the expression of AXIN2 protein substantially decreased, while β-catenin nuclear protein was up-regulated ($p < 0.01$) [Fig. 4a,b]. Results showed that AXIN2 silencing significantly attenuated the inhibitory effect of curcumin on $CD206^+$ cell proportions ($p < 0.001$) [Fig. 4c,d]. and increased the IL-10 and TGF-β levels ($p < 0.01$) [Fig. 4e,f]. These findings indicate that AXIN2 deficiency partially reverses curcumin's suppression of M2 polarization.

Curcumin Reverses the Inhibitory Effect of Macrophage-conditioned Medium on OSCC Cell Growth via the AXIN2/β-catenin Pathway

To determine whether the AXIN2/β-catenin pathway mediates macrophage regulation of OSCC cell growth, we generated AXIN2-knockdown macrophages, treated them with curcumin, and collected their conditioned medium. We examined the clonogenic and invasive abilities of the cells cultured in the conditioned media of different treatment groups. The Curcumin+Si-AXIN2 group showed a significant increase in colony formation in HSC3 and CAL33 cells compared to the Curcumin+Si-NC group ($p < 0.001$) [Fig. 5a–d]. Transwell assays further demonstrated that AXIN2 knockdown enhanced the invasiveness of HSC3 and CAL33 cells ($p < 0.01$) [Fig. 5e–h]. These results collectively indicate that inhibiting AXIN2 partially reverses the suppressive effect of curcumin-pretreated macrophage-conditioned medium on OSCC cell growth.

Discussion

The polarization state of macrophages is closely related to the progression of cancer. Among them, M2-type macrophages accelerate tumor invasion and metastasis through mechanisms such as secreting cytokines, promoting angiogenesis, and immune regulation [22]. This study is the first to confirm that curcumin can inhibit the M2 polarization of macrophages by regulating the AXIN2/β-catenin, thereby suppressing the growth and invasion of OSCC cells. This contributes significantly to understanding the anti-tumor mechanism of curcumin and the targeted treatment of OSCC.

Curcumin has been proven to have anti-tumor effects in various cancer models, including CRC [23], ovarian cancer [24] and non-small cell lung cancer [25]. Previous studies have mainly focused on the direct effects of curcumin on the proliferation, apoptosis, and drug resistance of tumor cells. However, its regulatory role on immune cells in the TME has gradually become a research hotspot. For instance, Deswal *et al.* [26] discovered that curcumin can inhibit the M2 polarization of macrophages by regulating the PI3K-AKT/STAT3 pathway, thereby weakening its ability to induce chemotherapy resistance in breast cancer cells to paclitaxel. Another study has shown that curcumin inhibits the development of CRC by regulating the polarization of M2 type macrophages [27]. This study further expanded this field, confirming the regulatory effect of curcumin on the polarization of macrophages in the microenvironment of OSCC and its influence on the secretion of inflammatory factors, thereby affecting the progression of OSCC.

Research evidence has confirmed that the polarization of M2-type macrophages is regulated by multiple signaling pathways. Among them, the aberrant triggering of the Wnt/β-catenin pathway has been proven to be closely related to the functional remodeling of macrophages [28]. As a key component of the β-catenin degradation complex, AXIN2 forms complexes, accelerating the degradation of β-catenin, thereby negatively regulating the activity of the pathway [29]. This study found that curcumin can upregulate AXIN2 in macrophages, inhibit the nuclear accumulation and transcriptional activity of β-catenin, which aligns with the findings of Huang *et al.* [30] in the liver cancer model that curcumin inhibits the β-catenin pathway and blocks the M2 polarization of macrophages by upregulating the expression of AXIN2. Furthermore, reducing the

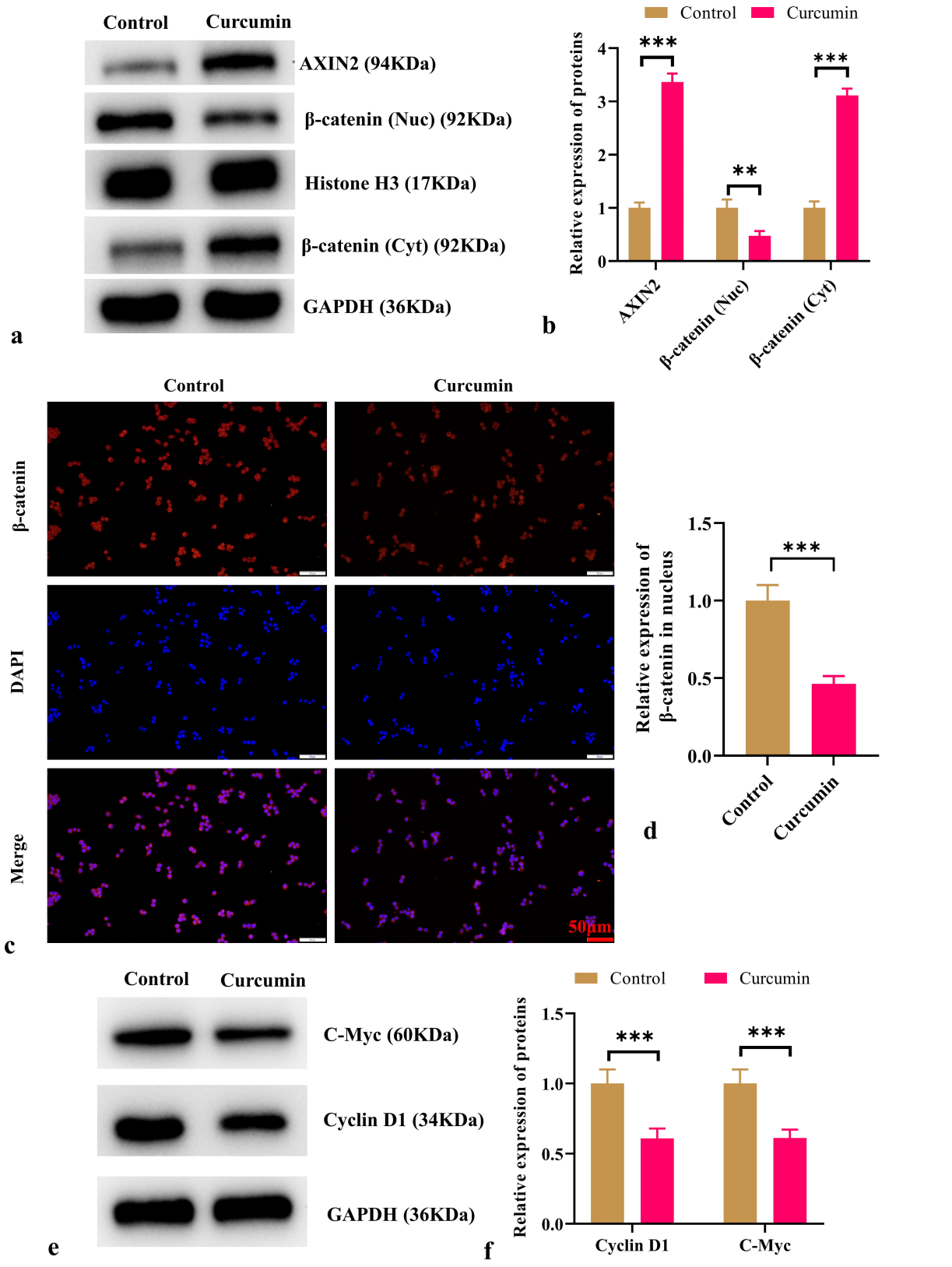

Fig. 3. Curcumin upregulates AXIN2 expression and suppresses β-catenin pathway activation in macrophages. (a,b) The expression of AXIN2 protein and β-catenin protein in macrophages was conducted using Western blotting after treatment with 20 μM curcumin. (c,d) The localization of β-catenin in macrophages was detected using immunofluorescence after treatment with 20 μM curcumin. (e,f) The expression levels of Cyclin D1 and c-Myc were detected in macrophages by Western blotting after treatment with 20 μM curcumin. n = 6; $^{**}p < 0.01$, $^{***}p < 0.001$.

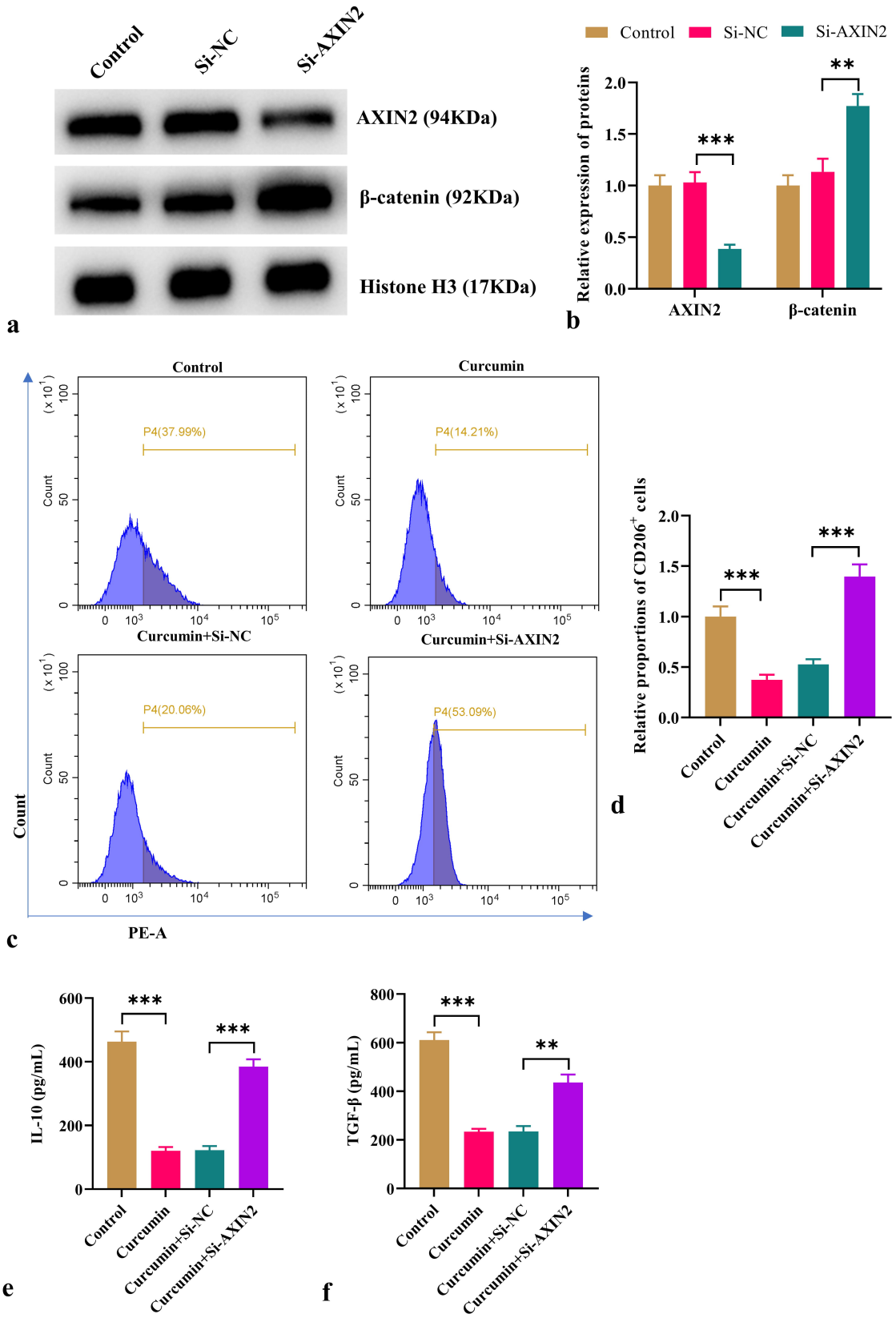

Fig. 4. AXIN2/β-catenin pathway is a critical mediator of curcumin-regulated macrophage polarization. (a,b) The expression of AXIN2 protein and β-catenin nucleoprotein knockdown was detected using Western blotting after AXIN2. (c,d) The M2 polarization marker CD163 in macrophages was detected by flow cytometry after AXIN2 knockdown. (e,f) The levels of IL-10 and TGF-β were detected by ELISA after AXIN2 knockdown. n = 6; $^{**}p < 0.01$, $^{***}p < 0.001$.

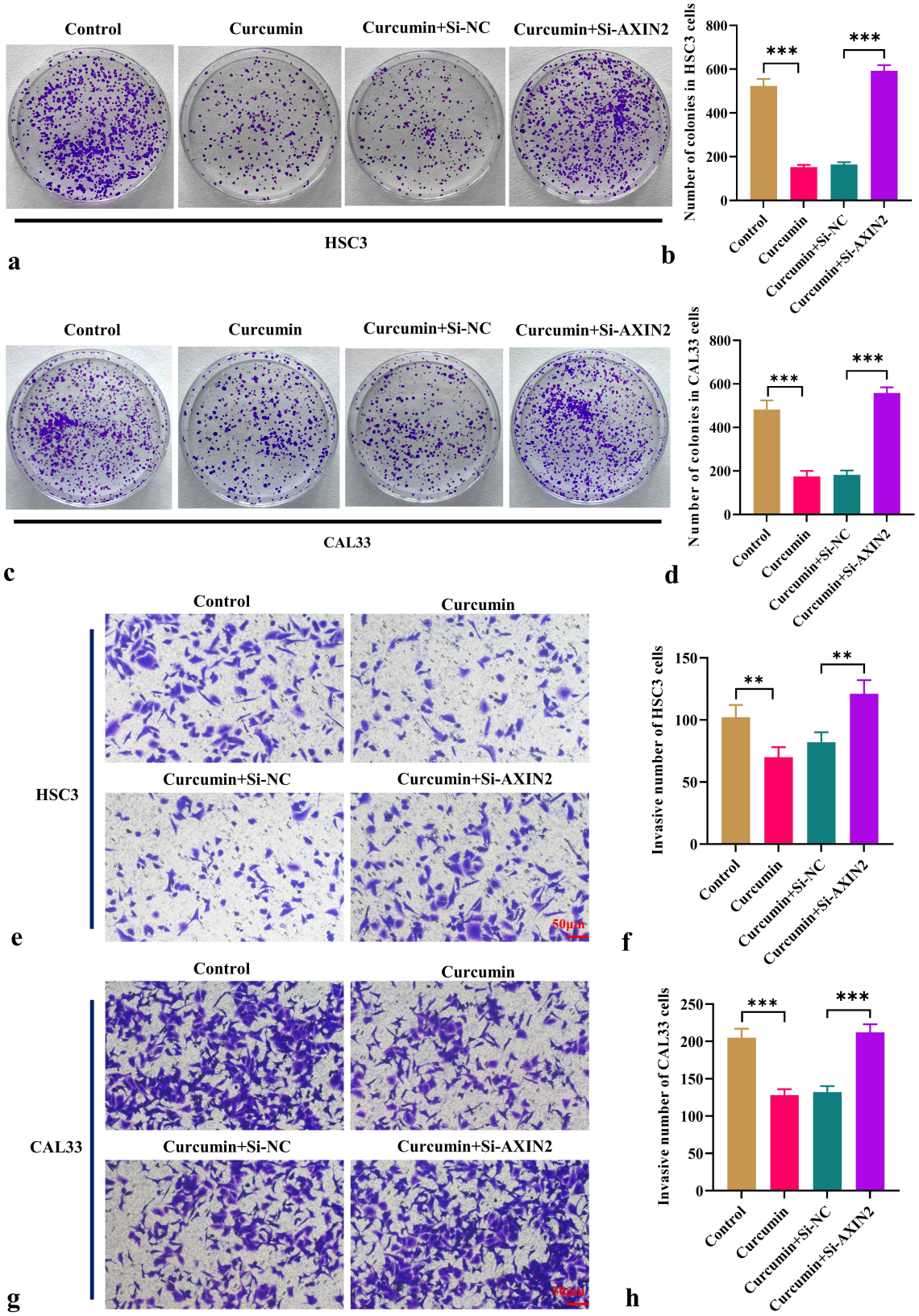

Fig. 5. Curcumin reverses the inhibitory effect of macrophage-conditioned medium on OSCC cell growth via the AXIN2/β-catenin pathway. (a,b) The proliferative capacity of HSC3 cells was assessed using colony formation assay after AXIN2 knockdown. (c,d) The growth potential of CAL33 cells was evaluated using colony formation analysis after AXIN2 knockdown. (e,f) The invasive capability of HSC3 cells was examined using Transwell assay after AXIN2 knockdown. (g,h) Transwell experiments were carried out to investigate the invasiveness of CAL33 cells after AXIN2 knockdown. n = 6; $^{**}p < 0.01$, $^{***}p < 0.001$.

expression of AXIN2 can partially reverse the suppressive effect of curcumin on M2 polarization, further confirming that AXIN2 is a crucial target for curcumin's action.

The malignant progression of OSCC depends on the dynamic interaction between tumor cells and the microenvironment. In this study, the conditioned medium of macrophages pretreated with curcumin suppressed the viability, colony formation and invasion ability of OSCC cells, and induced apoptosis. This is congruent with the results of Lin *et al.* [31], which revealed that inducing the polarization of M2-type macrophages and coordinating the paracrine glycoprotein non-metastatic protein B (GPNMB) can promote the migration and invasion of OSCC. It is worth noting that after knocking down AXIN2 in macrophages, the inhibitory effect of their conditioned medium on OSCC weakened, suggesting that curcumin regulates the secretion profile of macrophages via the AXIN2/β-catenin pathway and indirectly affects the behavior of tumor cells. This discovery reveals the key molecular mechanism in the "macrophage-tumor cell" cross-talk and provides experimental evidence for targeted therapeutic strategies targeting the tumor microenvironment.

This study elucidates the central role of the AXIN2/β-catenin pathway in curcumin's regulation of macrophage M2 polarization, supplementing the multi-target anti-tumor molecular mechanism of curcumin. Through conditional medium experiments and knockout validation, a complete regulatory chain of "curcumin inhibits OSCC progression" was established. This provides a new paradigm for understanding the mechanisms by which natural compounds exert anti-tumor effects by reshaping the immune microenvironment. However, the study still has limitations: only two OSCC cell lines—HSC3 and CAL33 were used *in vitro* experiments, and the conclusions should be verified in more cell models; in the future, animal models need to be included to further validate the suppressive function of curcumin on OSCC tumors and to deeply explore its molecular mechanism. In addition, the influence of curcumin on other immune cells in the TME (such as T cells and NK cells) can be further analyzed in the future through single-cell sequencing and other technologies to understand its overall regulatory effect on the immune microenvironment.

Conclusions

In summary, this study confirms that curcumin prevents the activation of β-catenin by upregulating AXIN2, thereby blocking macrophage M2 polarization, inhibiting inflammatory responses, and ultimately suppressing the malignant progression of OSCC.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

WWH, JSY and SM designed the research study. WLL, JRH and YPS performed the research. WWH, WLL and SM analyzed the data. All authors contributed to important 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

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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