J BONE ONCOL 46 (2024) 100601

Contents lists available at ScienceDirect

Journal of Bone Oncology

journal homepage: www.elsevier.com/locate/jbo

Research Paper

# Reversine inhibits proliferation and induces apoptosis of human osteosarcoma cells through targeting MEK1

Xianlong Chen [a], Yeyin Zhong [a], Simiao Wang [a], Shujie Xu [a,b], Junyuan Chen [c], Xin Cheng [a,d,*], Xuesong Yang [a,e,*]

[a] Division of Histology and Embryology, International Joint Laboratory for Embryonic Development & Prenatal Medicine, Medical College, Jinan University, Guangzhou 510632, China
[b] Department of Plastic and Cosmetic Surgery, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China
[c] Center for Bone, Joint and Sports Medicine, The First Affiliated Hospital of Jinan University, Jinan University, Guangzhou 510632, China
[d] Key Laboratory for Regenerative Medicine of the Ministry of Education, Jinan University, Guangzhou 510632, China
[e] Clinical Research Center, Clifford Hospital, Guangzhou 511495, China

## HIGHLIGHTS

- Systematic literature reviews suggested the anti-tumor potentials of reversine.
- Reversine inhibited the growth of osteosarcoma cell xenograft *in vivo*.
- Reversine inhibited osteosarcoma cells proliferation.
- Reversine induced osteosarcoma cell apoptosis.
- Reversine inhibited osteosarcoma cell migration.
- MEK1 is an effective target for reversine to inhibit the growth of osteosarcoma.

## ARTICLE INFO

*Keywords:*
Reversine
Anti-tumor effect
Osteosarcoma
MEK1
Systematic review

## ABSTRACT

Reversine, or 2-(4-morpholinoanilino)-6-cyclohexylaminopurine, is a 2,6-disubstituted purine derivative. This small molecule shows anti-tumor potential by playing a central role in the inhibition of several kinases related to cell cycle regulation and cytokinesis. In this study, systematic review demonstrated the feasibility and pharmacological mechanism of anti-tumor effect of reversine. Firstly, we grafted MNNG/HOS, U-2 OS, MG-63 osteosarcoma cell aggregates onto chicken embryonic chorioallantoic membrane (CAM) to examine the tumor volume of these grafts after reversine treatment. Following culture, reversine inhibited the growth of osteosarcoma cell aggregates on CAM significantly. *In vitro* experiment, reversine suppressed osteosarcoma cell viability, colony formation, proliferation, and induced apoptosis and cell cycle arrest at $G_0$-$G_1$ phase. Scratch wound assay demonstrated that reversine restrained cell migration. Reversine increased the protein expression of E-cadherin. The mRNA expression of Rac1, RhoA, CDC42, PTK2, PXN, N-cadherin, Vimentin in MNNG/HOS, U-2 OS and MG-63 cells were suppressed and PTEN increased after reversine treatment. Network pharmacology prediction, molecular docking and systematic review revealed MEK1 can be used as an effective target for reversine to inhibit osteosarcoma. Western blot results show the regulation of MEK1 and ERK1/2 by reversine was not consistent in different osteosarcoma cell lines, but we found that reversine significantly inhibited the protein expression of MEK1 in MNNG/HOS, U-2 OS and MG-63. All these suggested that reversine can exert its anti-tumor effect by targeting the expression of MEK1.

* Corresponding authors at: Division of Histology and Embryology, International Joint Laboratory for Embryonic Development & Prenatal Medicine, Medical College, Jinan University, Guangzhou 510632, China.
*E-mail addresses:* tchengxin@jnu.edu.cn (X. Cheng), yang_xuesong@126.com (X. Yang).

https://doi.org/10.1016/j.jbo.2024.100601
Received 19 November 2023; Received in revised form 3 March 2024; Accepted 15 April 2024
Available online 17 April 2024

## 1. Introduction

Osteosarcoma is the most prevalent primary bone malignancy, and its characteristic is that malignant mesenchymal cells can generate osteoid and immature bone [1]. Osteosarcoma is derived from primitive mesenchymal cells. The most frequent sites of osteosarcoma are the distal femur, the proximal tibia, and the proximal humerus, with 50 % of cases originating around the knee [1,2]. Osteosarcoma has a high propensity for local invasion and metastasis. Before the introduction of comprehensive chemotherapy, more than 90 % of osteosarcoma patients perished from pulmonary metastases. Osteosarcoma is typically resistant to conventional chemotherapy, meanwhile high-dose chemotherapy can cause severe adverse effects [3]. A low overall survival rate of osteosarcoma patients is attributable to drug resistance and early hematogenous dissemination in an inoperable setting. Despite the fact that the combination of surgery and chemotherapy has significantly improved the prognosis, the survival rate for patients with metastatic or recurrent osteosarcoma has been only 25 % over the past few decades [4]. Because of its high malignancy, the treatment of osteosarcoma has not been changed significantly over the past 30 years. Hence, there is an urgent need to develop new treatment strategies considering these obstacles in traditional treatment plans.

Reversine, a 2-(4-morpholinoanilino)-6-cyclohexylaminopurine analogue, was initially identified as a potential dedifferentiation drug which could reverse myoblasts to a progenitor-like state, and human dermal fibroblasts can also be dedifferentiated into progenitor cells [5]. The effect of reversine involves inhibitory activity on several kinases implicated in cell cycle regulation and cytokinesis in different cell types [6–8]. Based on these characteristics and its chemical nature, reversine is suggested as a potential anti-tumor drug. The double features of dedifferentiative and anti-tumor for reversine could be rationalized by its distinctive mechanism of action [9,10]. Recent studies have demonstrated that reversine can induce polyploidy, cell apoptosis and autophagy, and inhibit cell proliferation, invasion and metastasis, and it also exhibited significant cytotoxic effect on a variety of tumor cell lines *in vitro* [11–13].

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases that participate in gene expression, cell metabolism, proliferation, differentiation, and apoptosis in response to a variety of extracellular stimulus [14]. Classical MAPK signaling pathways include ERK1/2, p38s, JNKs, and ERK5, while non-classical MAPK signaling pathways include ERK3/4, ERK7/8, and NLK [15]. The ERK1/2 pathway is one of the most essential signaling pathways. MEK1/2 are phosphorylated and activated by RAF protein. Phosphorylated MEK1/2 (p-MEK1/2) phosphorylate ERK1/2 at T202/185 and Y204/187 [16]. Phosphorylated ERK1/2 (p-ERK1/2) can catalyze a variety of substrates, such as transcription factors (e.g., FOS, ETS, and MYC), protein kinases (e.g., RSK, MNK, and MSK), and apoptosis regulators (e.g., BIM and MCL1), to modulate various physiological processes [14,17]. MEK1 is responsible for transmitting signals from a variety of upstream kinases, it is the sole activator of downstream ERK. MEK has become an essential cancer treatment strategy due to its central position and significance [18].

In this study, we first conducted a systematic review of the current literatures on the anti-tumor effect of reversine, and then established an *in vivo* tumor growth model in chicken embryonic chorioallantoic membrane (CAM)[19]. The anti-tumor effects of reversine on the viability, proliferation, apoptosis, and migration of osteosarcoma cells *in vitro* were verified. The potential target gene of reversine was predicted using SwissTargetPrediction, STITCH and Similarity ensemble approach (SEA). After reversine molecular docking with MEK1, the putative mechanism of osteosarcoma growth inhibition by targeting MEK1 was elucidated by detecting the expression of MEK1 and ERK1/2 and their corresponding phosphorylation levels.

## 2. Material and methods

### 2.1. Systematic review

Methodology of systematic review complied with what were previously described [20]. After defining the research question and search strategy, PubMed (https://pubmed.ncbi.nlm.nih.gov/), Embase (https://www.embase.com/), Web of Science (https://www.webofscience.com/), Ovid (https://ovidsp.ovid.com/), and MEDLINE (https://www.medline.com) were used as the database to retrieve literatures published prio to April 2023, and the duplicate literature and those irrelevant to the keywords were excluded before the imported literatures were stored in the reference management software EndNote (version X9; Clarivate Analytics, London, UK), followed by screening the full text and determining selection criteria. Then draw the flow chart and identify the common characteristics from articles in final selection list. The detailed procedures of implementing the systematic review are shown in the results section of this article.

### 2.2. Cell culture

Human osteosarcoma cell lines: MNNG/HOS (Procell, CL-0492, Wuhan, CN), U-2 OS (Procell, CL-0236, Wuhan, CN) and MG-63 (BTCC, MG-63, Beijing, CN) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) with 10 % fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA), 1 % of penicillin–streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C and with 5 % $CO_2$.

### 2.3. Cell counting kit 8 (CCK8) assay

MNNG/HOS, U-2 OS and MG-63 cell viability were determined using CCK8 assays (Dojindo Molecular Technologies, Japan). All cells were cultured in 96-well plates ($2.5 \times 10^4$ cells/mL) and treated with different concentrations of reversine (1, 2, and 4 μM; Sigma-Aldrich, R3904, St. Louis, MO, USA). After 24 and 48 h culture, 10 μL of CCK8 (5 g/L) was added into the 96-well plates, followed by further incubation for 4 h at 37 °C. The CCK8 reactions were measured in Bio-Rad Model 450 Microplate Reader (Bio-Rad, Hercules, CA, USA) with 450 nm absorbance. Cell viability was indirectly determined using the ratio of the absorbance value for reversine-treated cells relative to the control cells (n = 6 for each group).

### 2.4. CAM xenograft of osteosarcoma cells

The Avian Farm of South China Agriculture University provided fertilized chicken eggs. Eggs were incubated at 38 °C for 7.5 days, and then windows were created in the air chamber. 100 μL of MNNG/HOS, U-2 OS or MG-63 cells aggregates with a density of $5 \times 10^7$ were transplanted onto the CAM using a pipette and confined by a silicone ring, followed by the addition of 50 μL Matrigel Matrix (Corning, 356234, Corning, NY, USA). On the 8.5th day added 50 μL 0.1 % DMSO (control; Sigma-Aldrich, St. Louis, MO, USA) or 50 μL reversine (1, 2, and 4 μM). The fertilized embryos containing MNNG/HOS xenoplants were incubated until the 14.5th day at 38 °C. Every 2 days, the medium inside the ring was changed. Photographs were taken on the 7th day after transplantation, using a stereomicroscope (Olympus MVX10, Tokyo, Japan). The harvested tumor xenoplants with CAM and attached blood vessels within the rings were fixed in 4 % paraformaldehyde (PFA) at 4 °C for 24 h. After the specimens were then dehydrated, cleared in xylene and embedded in paraffin, the samples were serially sectioned at 5 μm thickness on a microtome (Leica RM2126RT, Wetzlar, Hessen, Germany) and stained with hematoxylin and eosin dye (H&E; Sigma-Aldrich, St. Louis, MO, USA) for histological analysis. The formula ($0.52 \times length \times width \times height$) is used to calculate the tumor volume [21]. The length, width, height of xenoplants were quantitatively

analyzed using Image Pro-Plus 5.0 software (IPP; Media Cybernetics, Rockville, MD, USA). Each group had at least 3 samples analyzed.

### 2.5. Colony formation assay

MNNG/HOS cells at the logarithmic growth phase were digested and re-suspended in 10 % FBS-containing DMEM by single cells. Then the cells were plated at a density of 2000 cells/well in 6-well plates and cultured for 5 days. Next, different concentration of reversine was added and followed by further 24 and 48 h treatment. The cells were fixed by 4 % PFA and stained with 0.05 % crystal violet for 20 min at room temperature. Image J ( National Institutes of Health, Bethesda, MD, USA ) was used to analyze the number of colonies counted by measuring the staining area of crystal violet in each group.

### 2.6. Flow cytometry

MNNG/HOS cells were plated and cultured in DMEM containing 10 % FBS. After 24 and 48 h treatment with various concentrations of reversine or 0.1 % DMSO (control), the cells were harvested and then stained with the Annexin V-FITC kit (Annexin V-FITC Apoptosis Detection Kit; Beyotime, Shanghai, CN) according to the manufacturer's instructions. The samples were analyzed using a FACScan Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA). For cell cycle analysis, the fixed cells were incubated with 50 μg/mL propidium iodide (PI; BD Pharmingen, Franklin Lakes, NJ, USA), and then the cell cycle phase profile was determined by flow cytometry, and data were analyzed using ModFit software (Verity Software House, Augusta, ME, USA).

### 2.7. Immunofluorescent staining

MNNG/HOS cells from the control and reversine-treated groups were fixed by 4 % PFA and followed by immunofluorescent staining with polyclonal primary antibodies against PCNA (1:200; Abcam, ab18197, Cambridge, Cambs, UK) and Ki67 (1:200, Proteintech, 27309–1-AP, Chicago, IL, USA) overnight at 4 °C, The second antibody was Alexa Fluor 488 anti-rabbit IgG (1:1000, Cell Signaling Technology, 4412 s, Danvers, MA, USA). The sections were counterstained with 5 μg/mL of 4′-6-diamidino-2-phenylindole (DAPI; 1:1000, Invitrogen, 62248, Waltham, MA, USA), then photographed with an Olympus IX51 epifluorescence microscope (Leica DM 4000B, Tokyo, Japan). The Image J software was used to quantitatively analyze fluorescence intensity.

### 2.8. Western blotting

Using a radio-immuno-precipitation assay buffer (RIPA, Sigma-Aldrich, St. Louis, MO, USA) containing protease and phosphatase inhibitors, total protein was isolated from the DMSO (control) and reversine treatment groups. The concentration of the protein was measured using a BCA assay. Western blotting was implemented in the light of standardized procedures using specific antibodies: PCNA (1:1000; Abcam, ab18197, Cambridge, Cambs, UK), Ki67 (1:1000, Proteintech, 27309-1-AP, Chicago, IL, USA), Bax (1:1000, Cell Signaling Technology, 2772 s, Danvers, MA, USA), Bcl-2 (1:1000, Cell Signaling Technology, 3498 s, Danvers, MA, USA), E-cadherin (1:1000, immunoway, YT1454, Plano, TX, USA), MEK1 (1:1000, Cell Signaling Technology, 12671 s, Danvers, MA, USA), p-MEK1 (1:1000, Cell Signaling Technology, 9127 s, Danvers, MA, USA), ERK1/2 (1:1000, Cell Signaling Technology, 4695 s, Danvers, MA, USA) and p-ERK1/2 (1:1000, Cell Signaling Technology, 4370 s, Danvers, MA, USA). The internal control was β-actin (1:3000, Proteintech, 20536-1-AP, Chicago, IL, USA). Quantity One (Bio-Rad, Hercules, CA, USA) was used to acquire and analyze the chemiluminescent signals. All samples were performed in triplicate.

### 2.9. Scratch-wound cell migration

MNNG/HOS cells were plated into 6-well plates. A "wound" was created at 100 % confluence by scratching the monolayer cells with a 1 mL pipette tip. The cells were then rinsed with PBS and cultured in serum-free DMEM containing 1, 2, 4 μM reversine or 0.1 % DMSO (control). At 0, 12, and 24 h after scraping, images of the assays were captured using an inverted microscope (Nikon Eclipse Ti–U, Tokyo, Japan). Each cohort was evaluated in 3 separate wells, and the experiments were repeated at least three times.

### 2.10. RNA isolation and qPCR

Total RNA was extracted from MNNG/HOS, U-2 OS and MG-63 cells using a Trizol kit (Invitrogen, Waltham, MA, USA). RNA (1 μg) was reverse transcribed, using 1 μL Oligo dT, 1 μL StarScript II RT mix, primer, and 10 μL 2 × reaction mix, according to the manufacturer's instructions (Genstar, Beijing, China). First-strand cDNA (0.4 μL) was synthesized to a final volume of 20 μL using a SuperScript RIII first-strand kit (Invitrogen, Waltham, MA, USA). Following reverse transcription, PCR amplification of the cDNA was performed using human specific primers. The primers' sequences and the cited references are provided in Supplementary Table 3. The PCR reactions were performed in a Bio-Rad S1000TM Thermal cycler (Bio-Rad, Hercules, CA, USA). The expression of the genes was normalized to GAPDH, and the expression levels were compared by ΔΔCt. The qPCR results shown are representative of three independent experiments.

### 2.11. Network pharmacology applied to predict target proteins interacting with reversine and molecular docking

The candidate target proteins of reversine were identified using SwissTargetPrediction [22], STITCH [23], Similarity ensemble approach [24]. STRING, functional protein association networks [25], was used to generate a protein–protein interaction network among the target proteins. AutoDock software (AutoDock 4.2; Center for Computational Structural Biology, La Jolla, CA, USA) was used for molecular docking [26]. The structure of reversine was obtained from PubChem [27] and MEK1 from AlphaFold Protein Structure Database [28]. The final docking result was displayed using PyMOL software (Version 2.4; DeLano Scientific LLC, South San Francisco, CA, USA).

### 2.12. Data analysis

The statistical analysis was conducted using SPSS software (version 22.0; IBM, Armonk, NY, USA). GraphPad Prism (Version 9.0; GraphPad Software, San Diego, CA, USA) was utilized to construct statistical charts. All data were expressed as the mean value (Mean ± SD). The statistical analysis was completed with the ANOVA (Dunnett 2-tailed test) to determine if the data was a statistically significant difference between the control and reversine treatment groups. The data was considered statistically significant when $P < 0.05$ in all analyses. All statistical description and results were provided in the Supplementary Data.

## 3. Results

### 3.1. Systematic literature reviews suggested the anti-tumor potentials of reversine

To determine whether reversine is an effective anti-tumor compound, the literature retrieval was conducted in the databases of PubMed, Embase, Web of Science, Ovid, and MEDLINE using "reversine" AND ("tumor" OR "cancer") as search strategy. As a result, 294 articles were obtained, of which 254 were excluded after the first round of screening (including 179 duplicates and 75 articles with their titles and

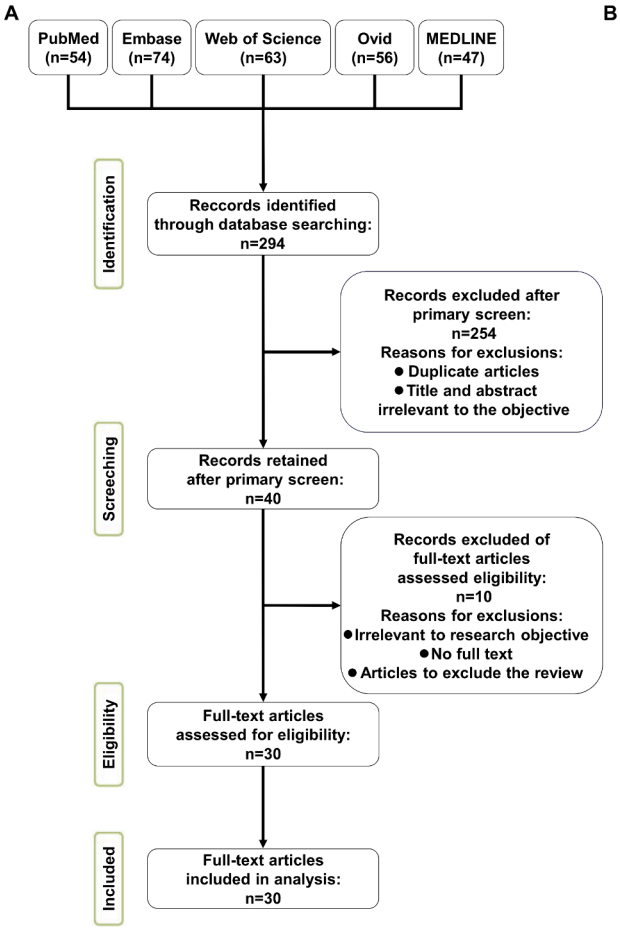
abstracts irrelevant to the objective). After 40 articles were attained according to the title and abstract, 10 articles were excluded for assessed eligibility (including 7 articles unrelated to the study of anti-tumor effect of reversine, 2 articles without full text and 1 review article). Finally 30 articles were selected after full text reading for assessed eligibility. (Fig. 1A). Subsequently, the conclusion from the study of anti-tumor effect of reversine shown in the 30 literatures were summarized and categorized (Fig. 1B). The results manifested that reversine has a significant anti-tumor effect on a variety of malignant tumor cell lines. These findings revealed by the systematic review indicated that reversine had a nature of anti-tumor and could be potentially used as an anti-tumor drug.

### 3.2. Reversine inhibited the growth of osteosarcoma xenograft in vivo

Human osteocsarcoma cell lines MNNG/HOS, U-2 OS and MG-63 were employed for the following studies. The results of CCK8 assay showed that 1, 2, 4 μM reversine treatment for 24 and 48 h significantly inhibited cell viability of MNNG/HOS (Fig. 2A-B), U-2 OS and MG-63 (Fig. S1A-B). We determined 1, 2, 4 μM as the concentration of reversine used for the subsequent experiments. Representative appearances of MNNG/HOS (Fig. 2C), U-2 OS and MG-63 (Fig. S1C) cells cultured after reversine treatment were shown. To determine whether reversine can inhibit the growth of osteosarcoma *in vivo*, the xenografts on CAM models were employed. As shown in Fig. 2D, MNNG/HOS, U-2 OS and MG-63 cells were inoculated onto the CAM of chicken embryos to grow into a spherical tumor-like tissue with the nutrients supplied by CAM vessels. After 7 days of incubation and reversine treatment, the results showed that reversine substantially inhibited the volume of MNNG/HOS (Fig. 2E), U-2 OS (Fig. S1E) and MG-63 (Fig. S1F) grafts in a concentration-dependent manner, while did not directly affect embryos' development and survival, suggesting that the anti-tumor effect of reversine was not directly associated with its influence on the host embryos (Fig. 2F).

### 3.3. Reversine inhibited osteosarcoma cells proliferation

After confirming that reversine inhibited the growth of osteosarcoma *in vivo*, we next examined the effects of reversine on the proliferation of osteosarcoma cells. The colony formation assay revealed that reversine inhibited osteosarcoma cell colony formation in a concentration-dependent manner (Fig. 3A-B). Utilizing flow cytometry, we found that the cell cycle of osteosarcoma cells treated with reversine for 12 and 24 h were arrested (Fig. 3C-D), with a tendency suspending at $G_0$-$G_1$ phase (Fig. 3C1-D3). Immunofluorescent staining revealed that reversine treatment for 24 and 48 h substantially inhibited the expression of




B

| | | Proliferation | Apoptosis | Autophagy | metastasis | polyploidy | tumor growth in vivo |
|---|---|---|---|---|---|---|---|
| 1 | Bijian K. et al, 2013 | ↓ | | | ↓ | | ↓ |
| 2 | Bosco B. et al, 2018 | ↓ | | | | ↑ | |
| 3 | Carlos J.A.E.G. et al, 2020 | ↓ | ↑ | ↑ | | ↑ | |
| 4 | Cheng L. et al, 2018 | ↓ | ↑ | | | ↑ | |
| 5 | D'Alise A.M. et al, 2008 | ↓ | ↑ | | | ↑ | |
| 6 | Fang C.Y. et al, 2018 | ↓ | | ↑ | | | |
| 7 | Hirakata C. et al, 2021 | ↓ | ↑ | — | | ↑ | |
| 8 | Hong O.Y. et al, 2022 | | | | ↓ | | |
| 9 | Hsieh T.C. et al, 2007 | ↓ | | | | ↑ | |
| 10 | Hu L. et al, 2021 | | | | | | ↓ |
| 11 | Hua S.C. et al, 2012 | ↓ | ↑ | | | ↑ | ↓ |
| 12 | Huang D. et al, 2019 | ↓ | ↑ | | | ↑ | |
| 13 | Jemaà M. et al, 2018 | — | — | | ↓ | ↑ | |
| 14 | Jemaà M. et al, 2012 | ↓ | ↑ | | | ↑ | |
| 15 | Kim J.S. et al, 2019 | ↓ | ↑ | | | | |
| 16 | Kuo C.H. et al, 2014 | ↓ | ↑ | | | ↑ | |
| 17 | Lee Y.R. et al, 2012 | ↓ | ↑ | ↑ | | ↑ | |
| 18 | Lima K. et al, 2019 | ↓ | ↑ | | | ↑ | |
| 19 | Lu C.H. et al, 2012 | | | ↑ | | | |
| 20 | Lu Y.C. et al, 2016 | ↓ | ↑ | ↑ | | ↑ | |
| 21 | Park Y.L. et al, 2019 | ↓ | ↑ | | | | |
| 22 | Piccoli M. et al, 2012 | ↓ | ↑ | | | ↑ | |
| 23 | Prajumwongs P. et al, 2021 | ↓ | ↑ | ↑ | | | |
| 24 | Qin H.X. et al, 2013 | ↓ | ↑ | | | ↑ | ↓ |
| 25 | Alves A. P. N. R. et al, 2016 | ↓ | ↑ | | | ↑ | |
| 26 | Xia P. et al, 2021 | ↓ | ↑ | | ↓ | | |
| 27 | Zhang Y. et al, 2022 | ↓ | ↑ | | ↓ | | |
| 28 | Zhu L. et al, 2020 | ↓ | ↑ | ↓ | | ↑ | |
| 29 | Buczkowicz P. et al, 2011 | ↓ | — | | | ↑ | |
| 30 | McMillin D.W. et al, 2006 | ↓ | ↑ | | | | |

REV: Reversine.
" ↑ " Promotional effect
"—" No significant change
" ↓ " Inhibitory effect

**Fig. 1. Systematic literature review summarizes the anti-tumor effects of reversine.** (A) In the PubMed, Embase, Web of Science, Ovid, MEDLINE medical database, the literatures were retrieved with "reversine" AND ("tumor" OR "cancer"). The inclusion and exclusion criteria and the retrieving process are shown in the flow chart. (B) According to the screening criteria, the conclusion from the study of anti-tumor effect of reversine shown in the selected literatures were summarized and categorized in the table. (Note: "↑" stands for the promotional effect under the relevant item confirmed by the study; "—" stands for the no significant change under the relevant item confirmed by the study; "↓" stands for the inhibitory effect under the relevant item confirmed by the study; Empty blank stands for not checking under the relevant item in the study. REV: reversine. (All the detailed summary of the literature is shown in the Supplementary Table 4).

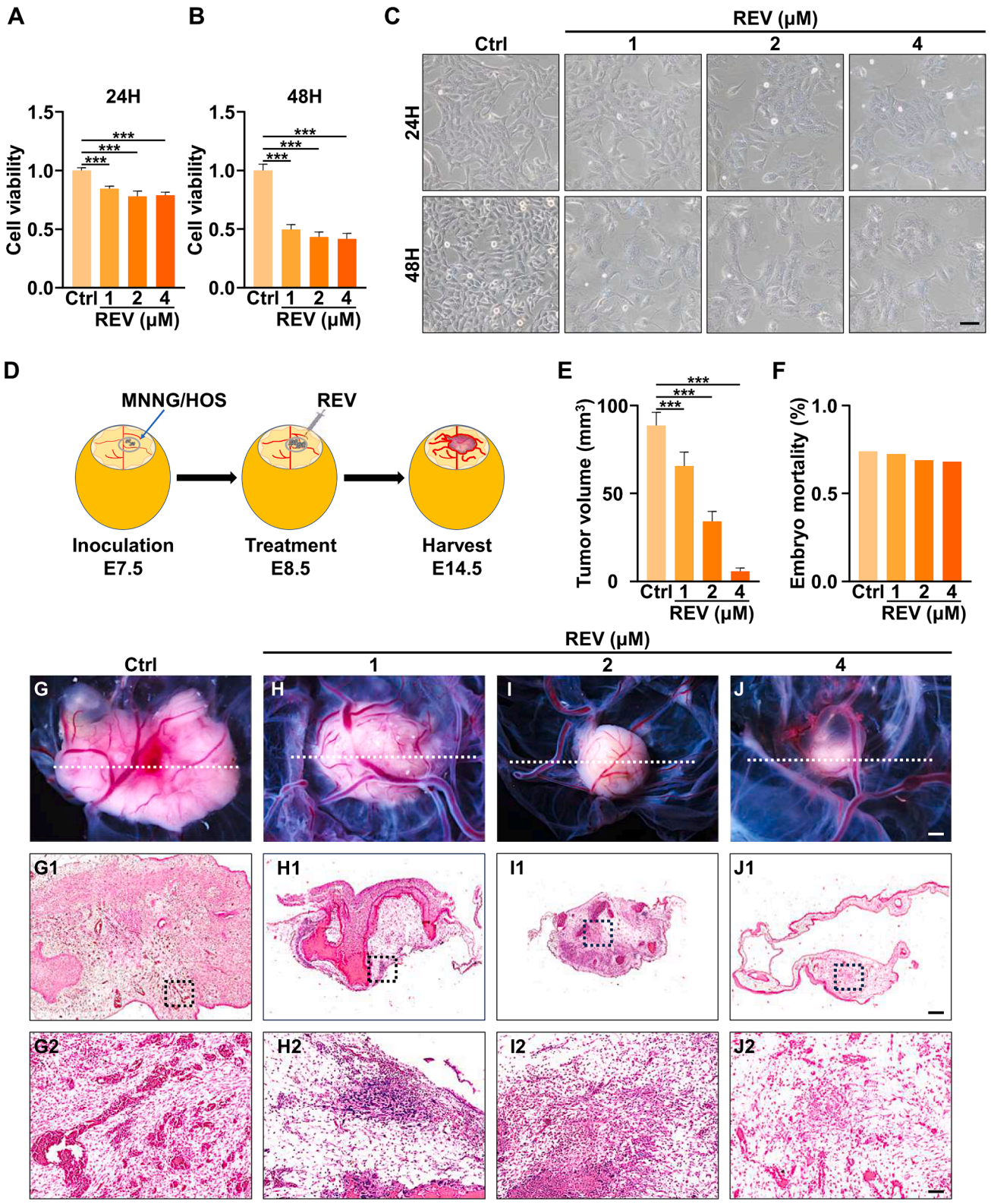



**Fig. 2. Assessing the viability of MNNG/HOS cells *in vitro* and growth of the MNNG/HOS cell mass xenograft on chicken embryonic CAM *in vivo* following reversine treatment.** (A-B) Bar charts showing the cell viability of MNNG/HOS after reversine treatment for 24 and 48 h measured by CCK8 assay. (C) Representative appearances of MNNG/HOS cells cultured after reversine treatment for 24 and 48 h. (D) Scheme illustrating how MNNG/HOS xenografts were transplanted onto the chicken embryonic CAM and treated with reversine. (E-F) Bar charts showing the comparison of the xenograft volumes (E) and embryo mortality (F) respectively among the control and reversine-treated groups. (G-J) Representative appearances of MNNG/HOS implants treated with 0.1 % DMSO (control, G) and 1, 2, 4 μM of reversine were shown respectively (H–J) on CAM. (G1–J1) Transverse sections of tissue taken from G–J at the level indicated by white dotted lines and stained with H&E. (G2-J2) High magnification images of G1–J1 (black dotted squares) were shown respectively. Scale bars = 200 μm in C, 1000 μm in G–J, 400 μm in G1–J1. 50 μm in G2-J2. *** $P < 0.001$, indicating significant difference between control and reversine treated groups (ANOVA). REV: reversine.

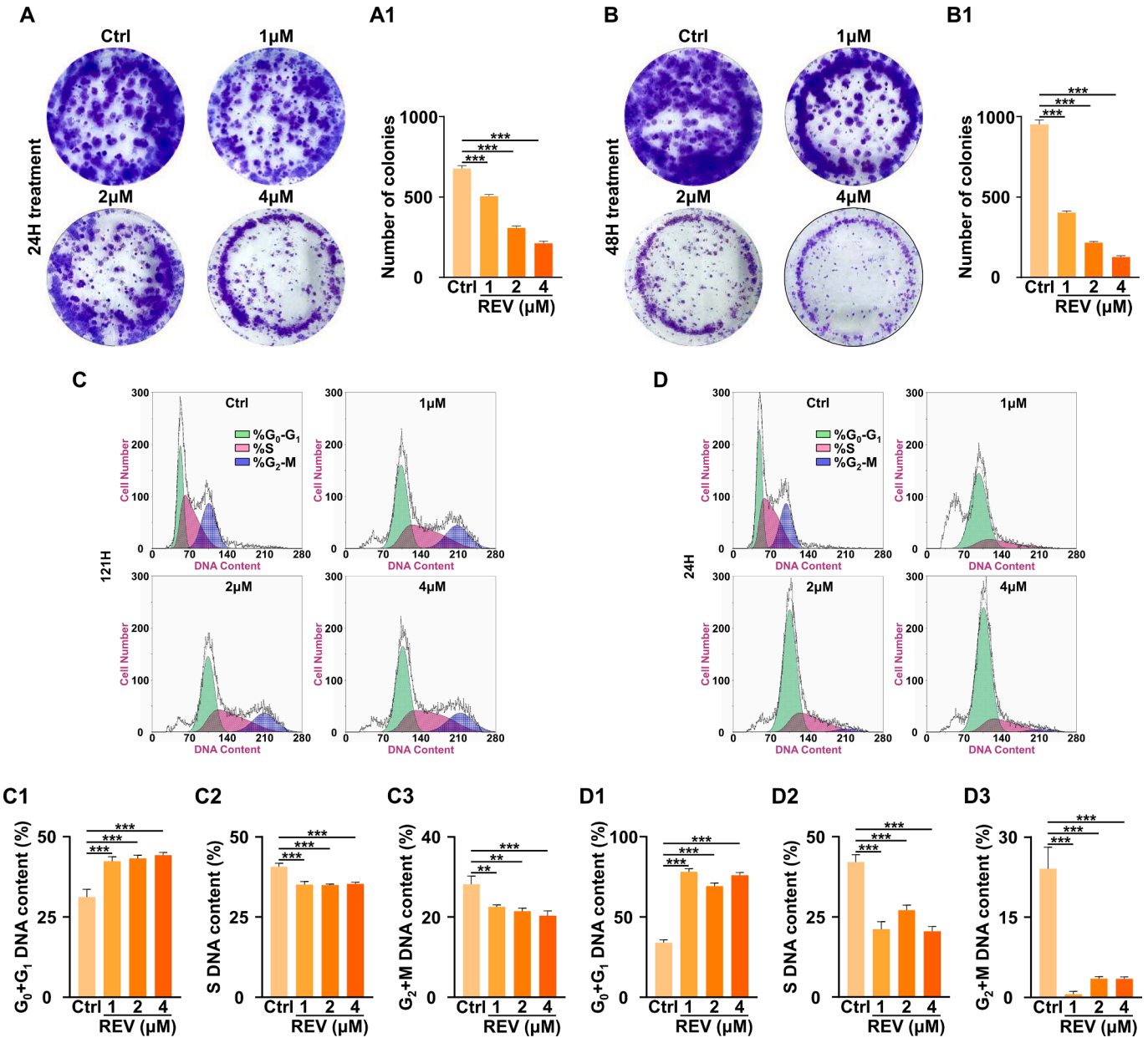



**Fig. 3. Assessing osteosarcoma cells colony formation and cell cycle following reversine treatment.** (A-B) Representative images of colony formation assays in MNNG/HOS cells following 5-day culture and treatment with reversine for 24 and 48 h were shown respectively. (A1-B1) Bar charts showing the comparison of the colony numbers among control and reversine-treated groups. (C-D) Flow cytometry assay showing the ratios of MNNG/HOS cells at different cell cycle stages in control and reversine-treatment groups for 12 (C) and 24 h (D). (C1-D3) Bar charts showing ratios of DNA content of $G_0$-$G_1$, S and $G_2$-M stage. ** $P < 0.01$ and *** $P < 0.001$, indicating significant difference between control and reversine treated groups (ANOVA). REV: reversine.

proliferation-related proteins PCNA and Ki67 in MNNG/HOS cells in a concentration-dependent manner (Fig. 4A-D). Western blot results also demonstrated that the expression of PCNA and Ki67 was inhibited by reversine in the same way (Fig. 4E-H). The protein expression of PCNA in U-2 OS and MG-63 cells were also inhibited after reversine treatment for 48 h (Fig. S2A, E). Taken together, we discovered that reversine substantially inhibited osteosarcoma cell proliferation.

### 3.4. Reversine induced osteosarcoma cell apoptosis

The data of flow cytometry demonstrated that osteosarcoma cells were substantially susceptible to apoptosis induced by reversine treatment for 24 and 48 h (Fig. 5A-B). Western blot analysis also revealed that reversine significantly induced the expression of pro-apoptotic protein Bax and inhibited the expression of anti-apoptotic protein Bcl-2 in a concentration-dependent manner (Fig. 5C-F; Fig. S2B-C, F-G). Our findings indicate that reversine can induce osteosarcoma cell apoptosis by activating Bax and inhibiting Bcl-2.

### 3.5. Reversine inhibited osteosarcoma cells migration

We performed scratch-wound assay to determine the influence of reversine on osteosarcoma cells migration. The results showed that cell migration from the edge to the midline of the "wound" was suppressed following reversine treatment for 12 and 24 h, in comparison with the controls (Fig. 6A). The area of wound closure was significantly reduced following reversine treatment (Fig. 6B1-B2). The results suggest that reversine inhibited the migratory ability of MNNG/HOS cells, and it was most obvious treated by 4 μM reversine.

E-cadherin is a tumor suppressor protein, and the loss of its

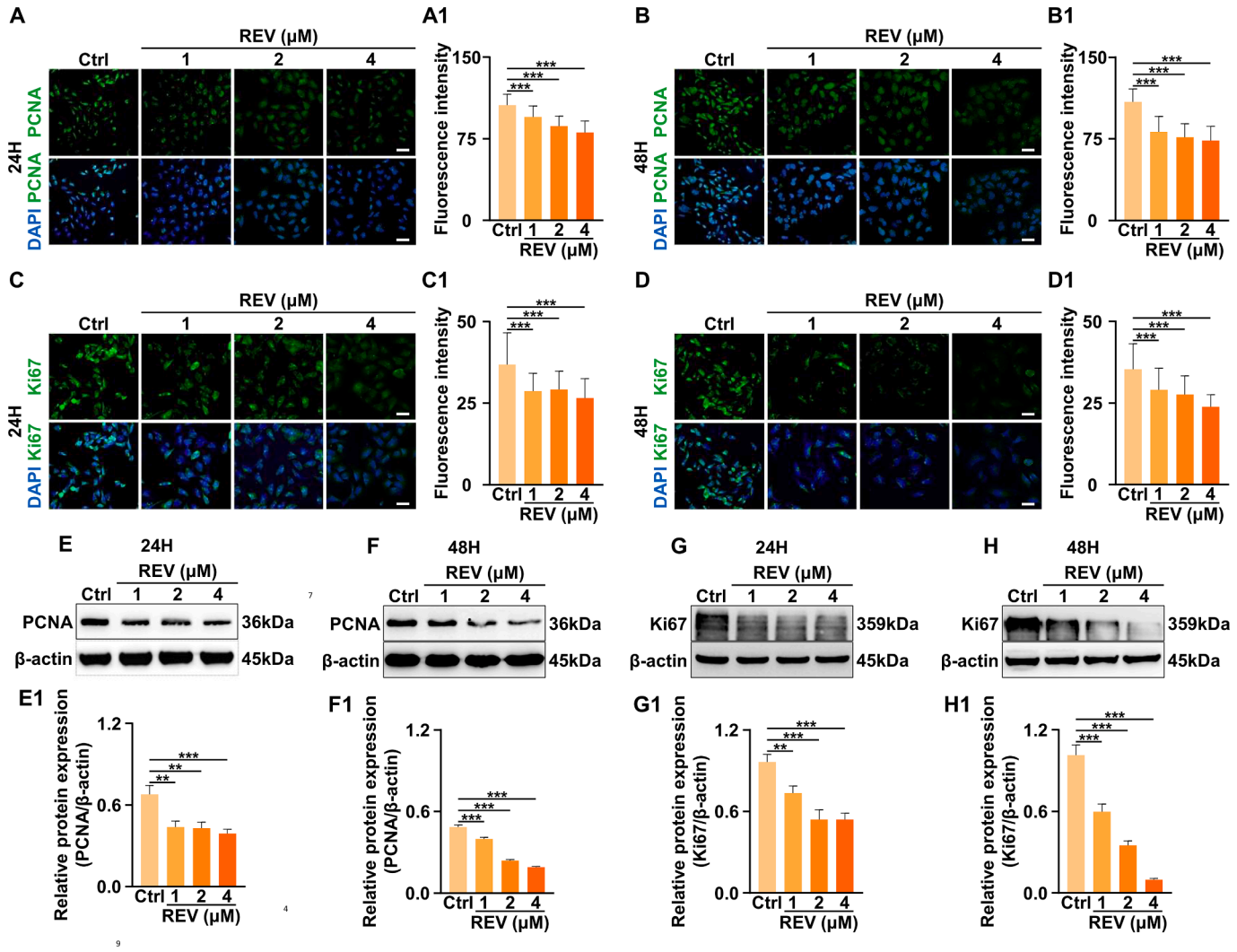



**Fig. 4. Assessing osteosarcoma cells proliferation following reversine treatment.** (A-D) Representative images of PCNA (A-B) and Ki67 (C-D) immunofluorescent staining on MNNG/HOS cells (counterstained with DAPI) in control and 24 or 48 h reversine-treated groups. Fluorescent staining intensities of PCNA (A1-B1) and Ki67 (C1-D1) of control and reversine-treated MNNG/HOS cells. (E-H) Western blot analysis of PCNA (E-F) and Ki67 (G-H) protein expression in MNNG/HOS cells treated with reversine for 24 and 48 h. (E1-H1) Bar charts showing the relative protein expression of PCNA (E1-F1) and Ki67 (G1-H1) in control and reversine-treated groups. Scale bars = 50 μm in A-D. ** $P < 0.01$ and *** $P < 0.001$, indicating significant difference between control and reversine treated groups (ANOVA). REV: reversine.

expression in association with the epithelial mesenchymal transition (EMT) occurs frequently during tumor metastasis. After reversine treatment, the protein expression of E-cadherin in MNNG/HOS (Fig. 6C-D), U-2 OS and MG-63 (Fig. S2D, H) increased in varying degrees. Rho-family small GTPases are a major convergence point of migration-associated signaling, The Rho family has several members, whose functions, in the context of migration, are represented by Rac1, RhoA, and CDC42. PTK2 and PXN are genes that encode focal adhesion kinase and Paxillin respectively, which reveals the importance and function of signaling complexes that localize the activity of Rho-family GTPases. Cells with elevated phosphatidylinositol 3,4,5-trisphosphate (PIP3) levels owing to loss of PTEN display a "migration" phenotype. Over-expression of E-cadherin or knockout of N-cadherin/Vimentin results in loss of metastatic potential. All in all, these genes play an important role in cell migration. The mRNA expression of Rac1, RhoA, CDC42, PTK2, PXN, N-cadherin, Vimentin in MNNG/HOS, U-2 OS and MG-63 cells were all suppressed in different degrees after reversine treatment. The mRNA expression of PTEN increased in MNNG/HOS, U-2 OS and MG-63 cells in various degrees. (Fig. 6E-L; Fig. S3).

### 3.6. *MEK1 was predicted to be the potential target for reversine*

SwissTargetPrediction, STITCH and Similarity ensemble approach (SEA) were used to predict the target of reversine, therefore investigating the underlying molecular mechanism. The top 25 targets of reversine were identified by SwissTargetPrediction, and the kinases has emerged as the majority of reversine's targets (Fig. 7A). STITCH further predicted that the targets of reversine should include MEK1, MEK2, MEK5 (Fig. 7B), and protein–protein interaction analysis among all predicted targets indicated that the targeting effect of reversine was closely associated with MAPK pathway (Fig. 7D). The results of the aforementioned three online prediction tools are enrolled to identify the common targets of reversine, generating results as MEK1 and TTK (Mps1) (Fig. 7C). The relationship between reversine and TTK has been described previously, therefore MEK1 would serve as the subject of this study. The structure of MEK1 was obtained from AlphaFold Protein Structure Database, while reversine's structure was obtained from PubChem. The binding energy of reversine's docking with MEK1 significantly exceeds the standard of docking energy in natural state, manifesting that reversine could be stably binding with MEK1

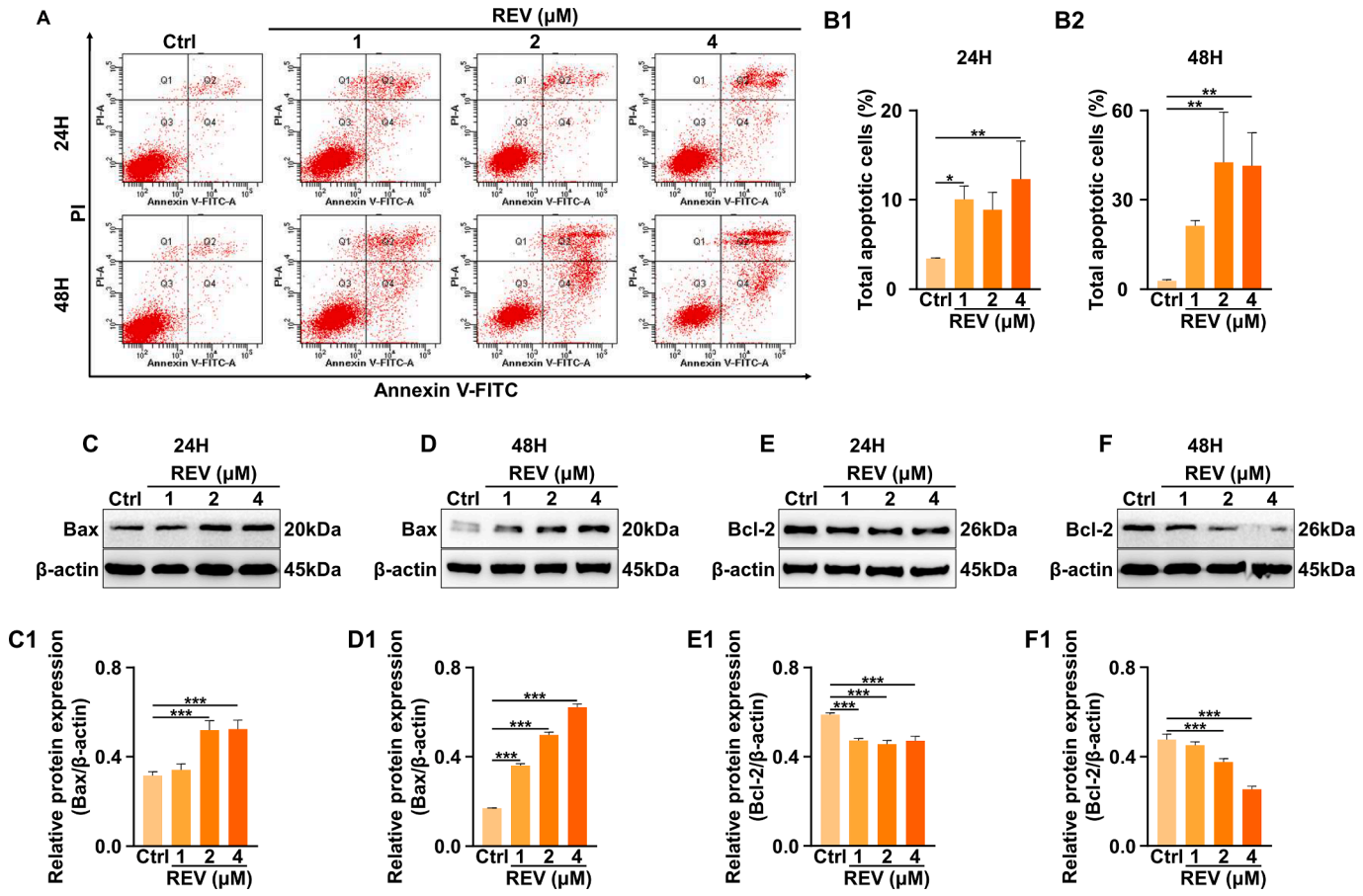



**Fig. 5. Assessing osteosarcoma cell apoptosis following reversine treatment.** (A) The apoptosis of MNNG/HOS cells were evaluated by flow cytometry with PI/Annexin-V double staining, after being treated with various concentrations of 1, 2, 4 μM reversine or DMSO for 24 and 48 h. (B1-B2) Bar charts showing the comparison of the ratios of apoptotic cells among control and reversine-treated groups. (C-F) Western blot analysis of Bax (C-D) and Bcl-2 (E-F) protein expression in MNNG/HOS cells treated with reversine for 24 and 48 h. (C1-F1) Bar charts showing the relative protein expression of Bax (C1-D1) and Bcl-2 (E1-F1) in control and reversine-treated groups. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, indicating significant difference between control and reversine treated groups (ANOVA). REV: reversine.

(Supplementary Table 13). The conformation of the lowest binding energy is displayed by PyMol (Fig. 7E1-E2).

### 3.7. *MEK1 was proved to be a potential target for reversine*

To investigate whether targeted MEK1 can effectively inhibit the growth of osteosarcoma, also because MEK1 is the sole activator of downstream ERK1/2, we firstly did literature retrieval by using "osteosarcoma" AND "MEK1-ERK1/2″ as searching strategy in the databases of PubMed, Embase, Web of Science, Ovid, and MEDLINE. As a result, 349 articles were obtained, of which 308 were excluded after the first round of screening (including 75 duplicates and 233 articles with their titles and abstracts irrelevant to the objective). After 41 articles were attained according to the title and abstract, 13 articles were excluded for assessed eligibility (including 8 articles unrelated to the study of inhibition of osteosarcoma growth by targeting MEK1, 4 articles without full text and 1 withdrawn article). Finally, 28 articles were selected after full text reading for assessed eligibility (Fig. S4A). Next, the conclusion from the study of targeted MEK1 effect on the inhibition of osteosarcoma growth shown in the 28 literatures were summarized and categorized (Fig. S4B-C). Their anti-tumor effects were involved in inhibiting proliferation and metastasis, inducing apoptosis and autophagy, but the expression of MEK1-/ERK1/2 were not identical. Lastly, Western blots was used to verify the changes of MEK1-ERK1/2 pathway in reversine-treated MNNG/HOS, U-2 OS and MG-63 cells. The results showed that the regulation of reversine on the expression and phosphorylation of MEK1 and ERK1/2 proteins in different osteosarcoma cell lines were not consistent, but the protein expression of MEK1 was inhibited in different degrees in all osteosarcoma cell lines. The expression of p-ERK1/2 was significantly enhanced in MNNG/HOS and p-MEK1 in U-2 OS respectively. (Fig. 8; Fig. S5).

## 4. Discussion

As the most common primary bone malignancy, osteosarcoma has posed significant challenge to the treatment and survival rate improvements of the patients worldwide [29]. Conventional chemotherapy, relying on the same drugs as it has done since early 1980 s, often proves ineffective due to resistance and severe side effects. The high heterogeneity of osteosarcoma cells further complicates the development of targeted therapies [30]. Therefore, there is an urgent need for innovative therapeutic approaches for osteosarcoma (Fig. 9).

Reversine can induce dedifferentiation and inhibit multiple kinases involved in the regulation of the cell cycle and cytokines. These properties suggest its potentials as an anti-tumor drug. Reversine has been shown to selectively kill cancer cells [10], induce apoptosis in a time- and dose-dependent manner [31–34], and promote autophagy [35–37]. It has exhibited cytotoxicity across various malignant tumor cell lines and inhibited tumor progression and metastasis *in vivo* [38,39]. Importantly, reversine has also been proved to inhibit tumor stromalization, and the activity of reversine was reflected in the cellular interactions with the microenvironment as an anti-tumor agent, thereby preventing

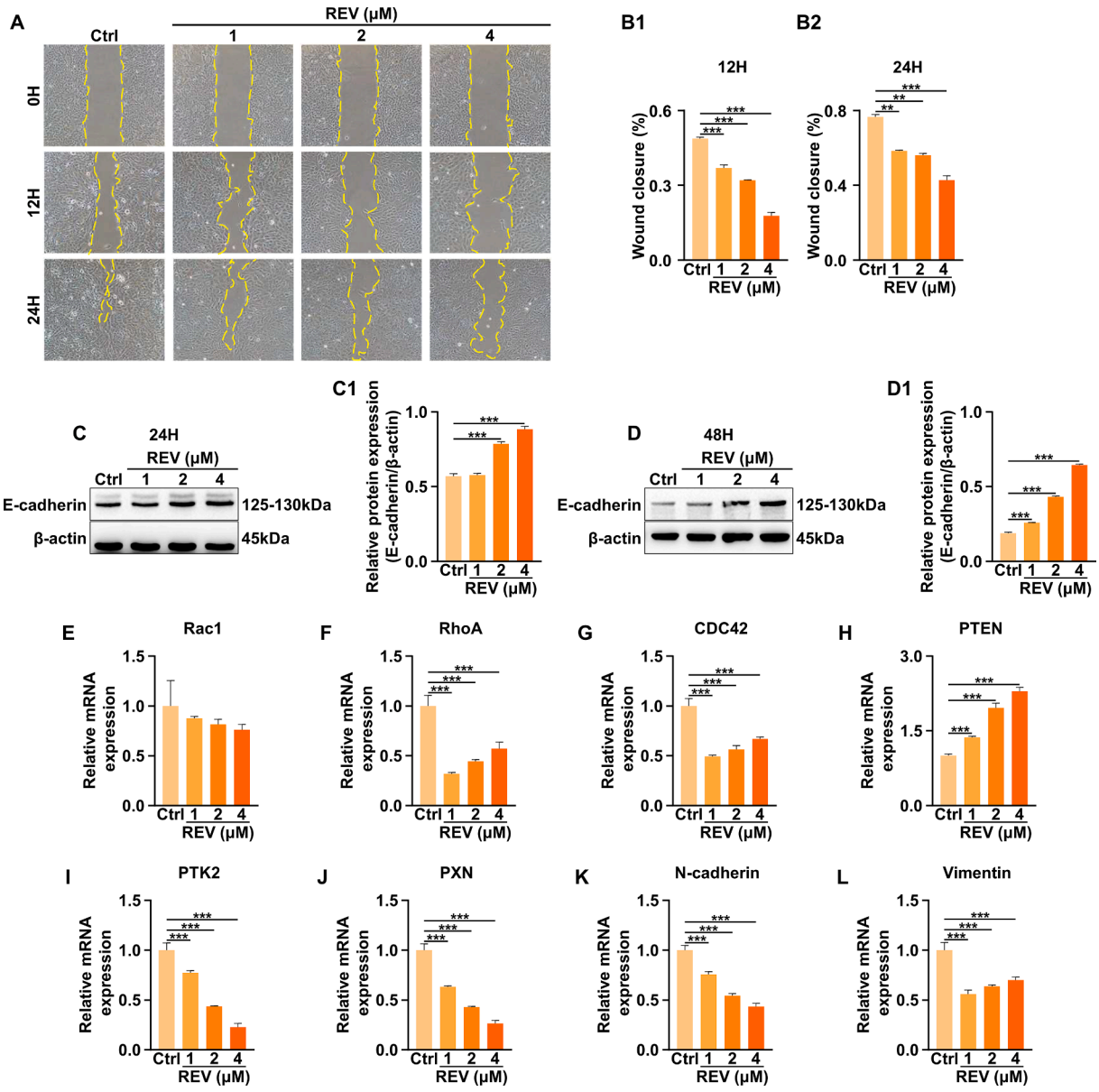



**Fig. 6. Assessing osteosarcoma cell migratory abilities following reversine treatment.** (A) Representative bright-field images of MNNG/HOS cells migration in scratch wound assays of control and reversine-treated groups at 0, 12, and 24 h after reversine treatment. (B1-B2) Bar charts showing the comparison of the wound closure of MNNG/HOS among the different groups. (C-D) Western blot analysis of E-cadherin protein expression in MNNG/HOS cells treated with reversine for 24 and 48 h. (C1-D1) Bar charts showing the relative protein expression of E-cadherin in control and reversine-treated groups. (E-L) Bar chart showing the relative mRNA expression of genes related to migration in MNNG/HOS cells.

tumor formation [40]. Osteosarcoma, characterized by high vasculacture, heavily relies on neovascularization for growth and spread [41]. Our previous work has indicated that reversine could inhibit osteosarcoma growth by supressing tumor angiogenesis [42]. Another study suggested that reversine could induce apoptosis of MG-63 osteosarcoma cells *via* mitochondria-mediated intrinsic pathway and death receptor-mediated extrinsic pathway [43]. Still, the specific molecular mechanisms underlying reversine's effect on osteosarcoma cells need to be further investigated.

In this study, the systematic review confirmed that reversine is an effective anti-tumor compound with a variety of biological mechanisms. Reversine effectively inhibited the osteosarcoma cell viability. Using an *in vivo* tumor growth model on CAM of chicken embryos, we displayed that the tumor sizes decreased significantly following reversine treatment. Furthermore, reversine substantially inhibited the colony formation of osteosarcoma cells, and arrested the cell cycle of osteosarcoma cells in $G_0$-$G_1$ phase. Reversine induced apoptosis by activating Bax and inhibiting Bcl-2, consistent with previous findings [43].

Aurora kinase B (AURKB) has been identified as a crucial factor in cell cycle regulation and cytokinesis, making it one of the known targets of reversine. Reversine was found to significantly inhibit AURKA and AURKB [6], suggesting that it may disrupt cytokinesis and induce mitotic abnormalities in tumor cells by decreasing AURKB activity. As a downstream of AURKB, protein TTK (Mps1) plays a central role in spindle assembly, error correction and cytokinesis, and it is proposed as another target for reversine, although TTK has a higher affinity and selectivity for reversine than AURKB [7,8].

The prediction of MEK1 as a target for reversine led to further investigation. The action of reversine was closely related to the MAPK pathway, and molecular docking results confirmed the stable interaction between reversine and MEK1. The targeted MEK1-ERK can effectively inhibit osteosarcoma growth, but the regulation of MEK1-ERK1/2 varied

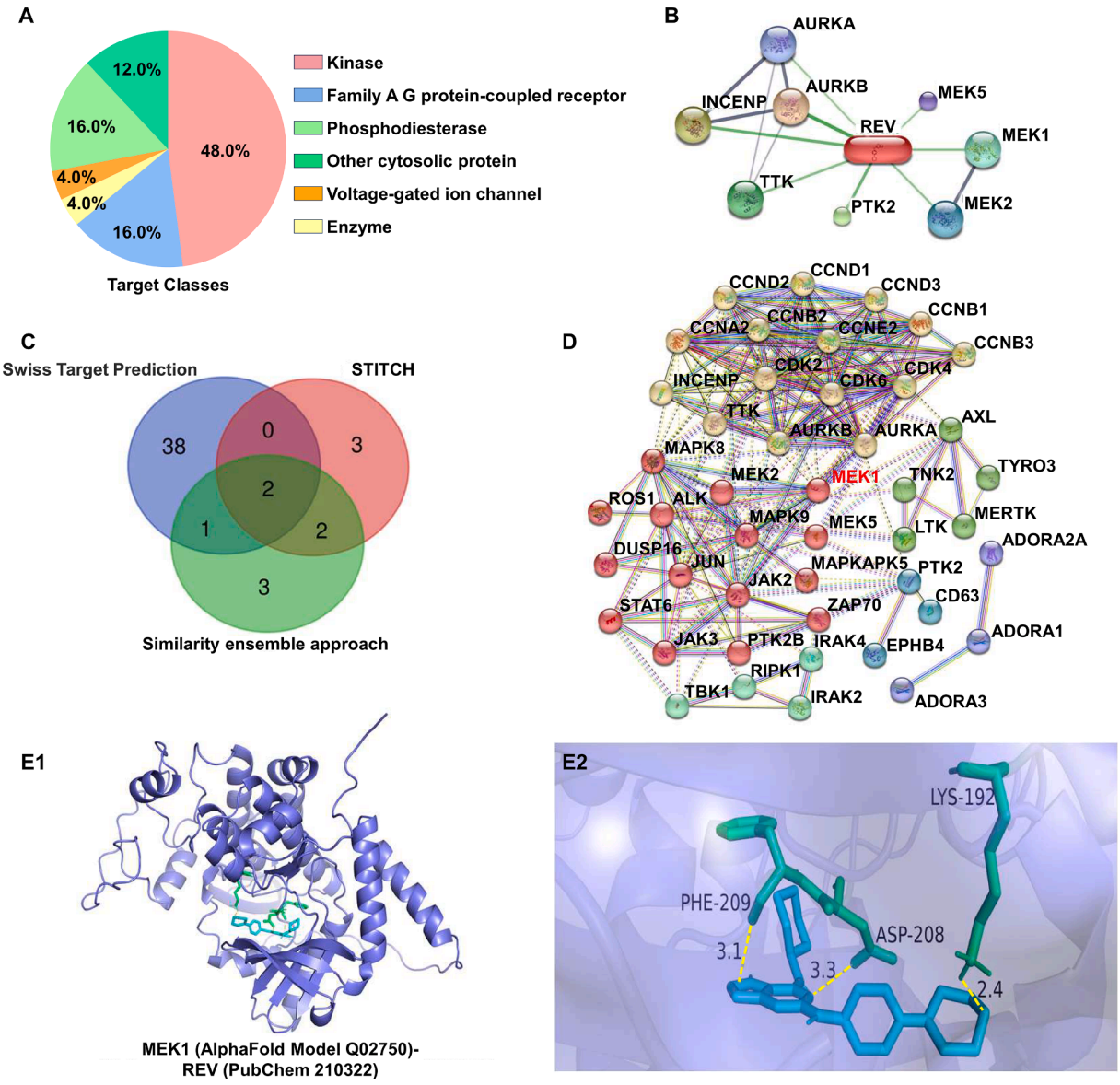



**Fig. 7. Determining the target genes of reversine using network pharmacology and enrichment analysis and the results verified by molecular docking.** (A) The pie chart of SwissTargetPrediction shows the classification of the top 25 targets of reversine. (B) STITCH: chemical association networks shows the interaction network among reversine and the target proteins. (C) The Venn diagram shows the intersection of the predictions of SwissTargetPrediction, STITCH: chemical association networks and Similarity ensemble approach. TTK and MEK1 emerge as the intersectional part of the three databases. (D) STRING generates a protein–protein interaction network among the predicted target genes of reversine, which are divided into six clusters according to the function of proteins. Target proteins represented by bubbles of the same color indicate that functional correlation among them exist, and MEK1 is emphasized in red. (E1) PyMol displays the conformation with the lowest binding energy in the docking result. (E2) Enlargement of the conformation, purple substance represents the protein structure of MEK1, cyan substance represents the chemical structure of reversine, green substance represents the amino acid residue sites that bind reversine. The yellow dotted line and numerical value represent the binding hydrogen bond and binding energy. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

under different treatment conditions. The regulation of reversine on the expression and phosphorylation of MEK1 and ERK1/2 proteins in different osteosarcoma cell lines were not consistent, this may be due to the high heterogeneity of osteosarcoma cells. But the protein expression of MEK1 was inhibited in different degrees in all osteosarcoma cell lines. In a previous report using C2C12 myoblasts, the results showed that 20 nM reversine blocked MEK1-dependent signal transduction by measuring the phosphorylation of ERK1/2, indicating that MEK1 inhibition was required for reversine dedifferentiation, MEK1 was identified as putative targets of reversine [44]. However, there has been no previous evidence of reversine's specific targeted inhibition of MEK1 in tumor cells. Others examined the *in vitro* effect of reversine on 26 kinases, and reported that reversine inhibited the activity of MEK1, the IC50 for MEK1 was greater than 1.5 μmol/L [6]. Functioning as a critical regulator of cell proliferation, differentiation, cell cycle, apoptosis, and metastasis, MEK1 holds significant promise for potential anti-tumor target [14,45]. Several MEK1 inhibitors are currently in various stages of preclinical and clinical testing, due to its central function in tumorigenesis, tumor maintenance, and metastasis.

The RAF-MEK-ERK signaling cascades modulate fundamental physiological functions through ERK activity, and ERK phosphorylates a vast array of substrates with diverse molecular functions. This pathway is implicated in various human malignancy, with abnormalities in genetic, transcriptional, and post-translational aspects [46]. Dysregulation of RAF-MEK-ERK pathway contributes to tumor proliferation, survival, invasion, metastasis, extracellular matrix degradation, and angiogenesis

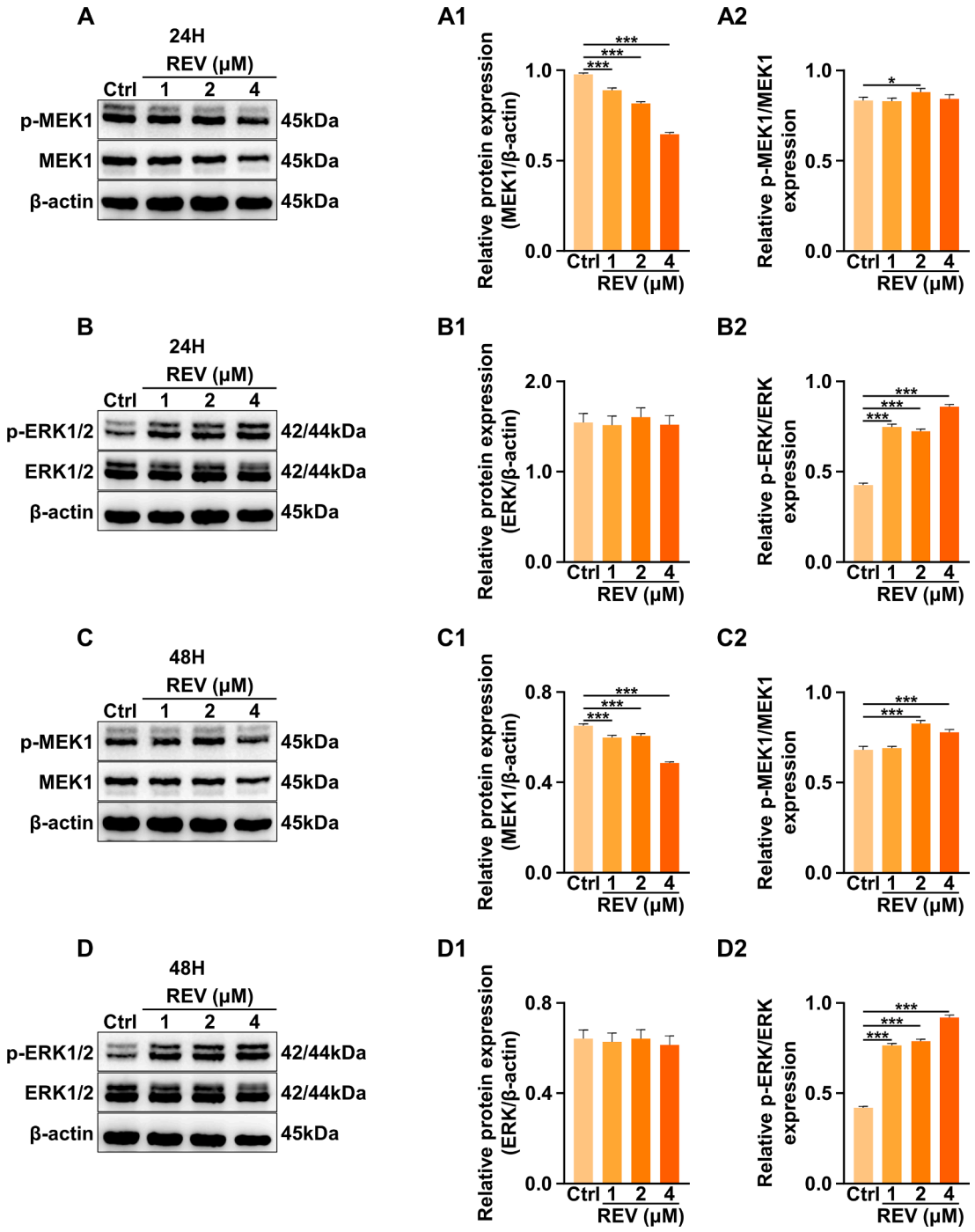



**Fig. 8. Assessing MEK1 and downstream ERK1/2 phosphorylation level and protein expression in MNNG/HOS cells following reversine treatment.** (A-D) Western blot analysis of p-MEK1, MEK1 and p-ERK1/2, ERK1/2 protein expression in MNNG/HOS cells treated with reversine for 24 and 48 h. (A1-D1) Bar charts showing the relative protein expression of MEK1 and ERK1/2 in MNNG/HOS cells treated with reversine for 24 and 48 h. (A2-D2) Bar charts showing the relative p-MEK1/MEK1 expression and relative p-ERK/ERK expression in control and reversine-treatment groups. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, indicating significant difference between control and reversine treated groups (ANOVA). REV: reversine.

[45]. The activation of this pathway is often observed in malignant tumors.

It's noteworthy that some substances with anti-tumor activity can inhibit the growth of osteosarcoma by activating ERK. For instance, chelerythrine activates ERK1/2 to induce apoptosis of osteosarcoma cells in a dose- and time-dependent manner [47]; 1α,25-dihydroxyvitamin $D_3$ continuously activates ERK1/2 signal transduction, leading to up-regulation of c-Jun/Fos (AP-1) complex and subsequent regulation of $P21^{waf1}$ expression [48]. Ganglioside GD1a can inhibit the growth of murine FBJ osteosarcoma cells by activating ERK1 phosphorylation. The aforementioned studies showed that the inhibition of osteosarcoma growth can be accomplished by activating ERK rather than inhibiting it [49–51]. Despite that MEK-ERK signaling cascades are activated through stepwise specific phosphorylation, Sorafenib modulates MEK and ERK in a inconsistent way in exerting anti-osteosarcoma effect [52], which reflected the intricate nature of these pathways. That is, while reversine exerts an anti-tumor effect, the expression and phosphorylation of MEK1 and ERK1/2 are not consistent in different osteosarcoma

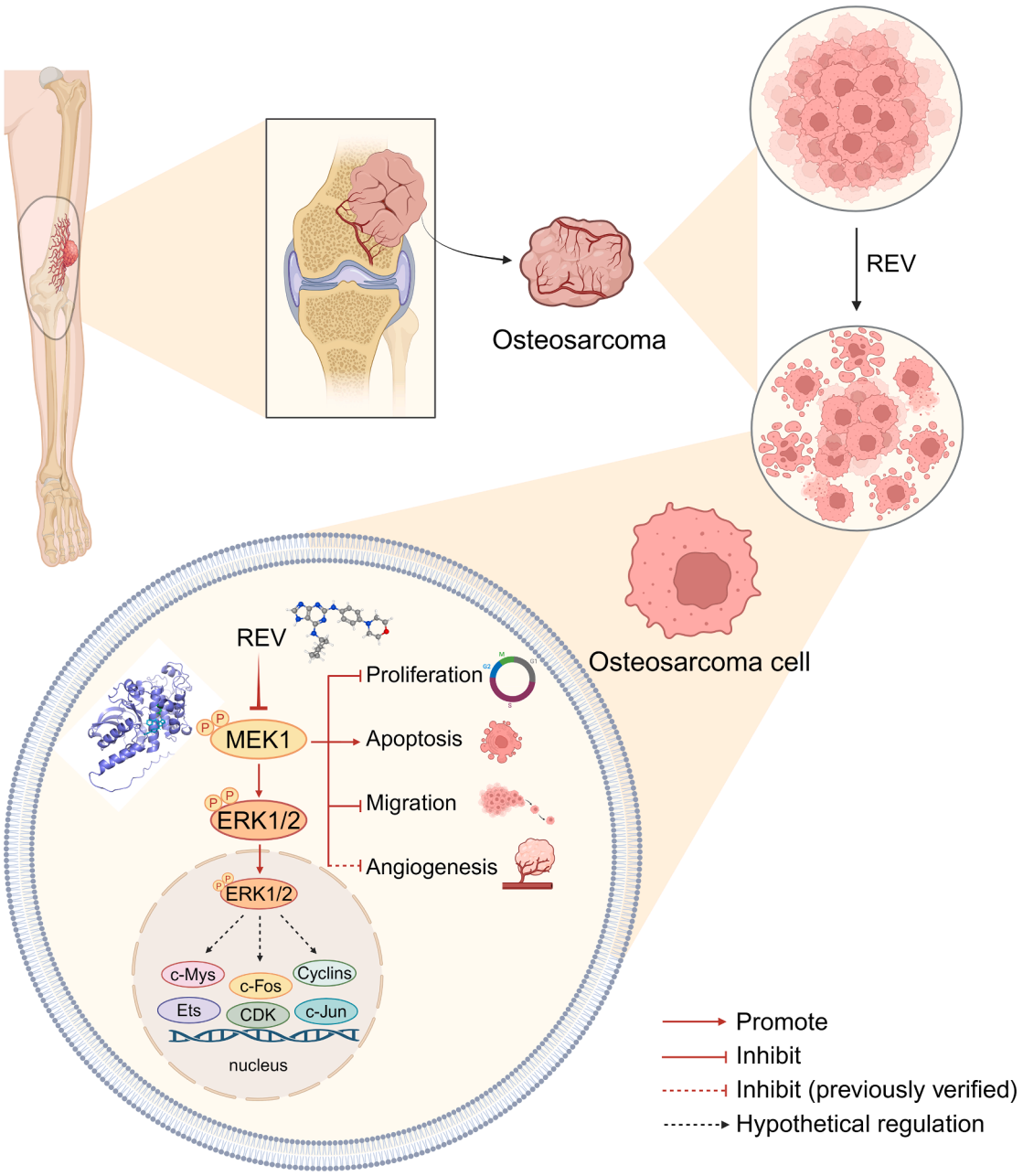



**Fig. 9. Proposed mechanism of reversine's inhibiting osteosarcoma growth by targeting MEK1.** This figure is created with BioRender.com.

cell lines.

## 5. Conclusion

To sum up, the experimental results in this study emphasize the potential anti-tumor activity of reversine. In this new discovery, the effect of reversine on the expression of MEK1 in tumor cells was detected for the first time. Reversine showed anti-tumor activity by inhibiting proliferation and arresting cell cycle in $G_0$-$G_1$ phase, inducing apoptosis and inhibiting metastasis in MNNG/HOS, U-2 OS and MG-63 cell lines. We found that reversine significantly inhibited the protein expression of MEK1 in MNNG/HOS, U-2 OS and MG-63 cells. All these suggested that reversine can exert its anti-tumor effect by targeting the expression of MEK1. However, its exact molecular mechanism needs to be further studied. Reversine is expected to be a candidate drug for the treatment of osteosarcoma in the future.

## Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Institutional Animal Care and Use Committee (IACUC) of Jinan University (IACUC-20181126-02, 26 November 2018).

## Funding

This study was supported by Natural Science Foundation of Guangdong Province [2021A1515012393], NSFC grant [82371692] and Guangdong Medical Science and Technology Research Foundation [A2023145], the Fundamental Research Funds for the Central Universities [21623311], Guangzhou Basic and Applied Basic Research [SL2024A04J00960], and College Students Innovation and Entrepreneurship Training Program [CX23303]. We would like to thank Experimental Technology Center and Medical Experimental Center of Jinan

University.

## CRediT authorship contribution statement

**Xianlong Chen:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Yeyin Zhong:** Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Simiao Wang:** Software, Methodology, Investigation, Formal analysis, Data curation. **Shujie Xu:** Software, Methodology. **Junyuan Chen:** Methodology, Funding acquisition. **Xin Cheng:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization. **Xuesong Yang:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbo.2024.100601.

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