Original Article
Modulation of anoikis resistance in MG63 osteosarcoma cells by sclareol via inhibiting Ezrin/Fak expression

Jianwen Mo, Renze Yang, Daifen Zhang

Department of Orthopedics Surgery, The First Affiliated Hospital of Gannan Medical University, Ganzhou, Jiangxi, China

Abstract: Osteosarcoma is one primary bone tumor with high risk of blood borne metastasis, thus causing aggravation and patient death. The study of molecular mechanism underlying metastasis of osteosarcoma thus may help to relieve the threat of osteosarcoma. Sclareol has been found to have anti-tumor effects in leukemia, colorectal cancer and osteosarcoma, but with unclear functional mechanism so far. Therefore we treated MG63 osteosarcoma cells with sclareol, to observe the condition of anoikis in cells, in an attempt to investigate the modulation of osteosarcoma cell anoikis by sclareol. MG63 cells were cultured by poly HEMA approach. CCK8 reagent was used to detect the cytotoxicity of sclareol on suspended MG63 cells, in order to determine the optimal dosage of sclareol. MG63 cells were further divided into DMSO and sclareol group, in which real-time florescent quantitative PCR and Western blotting were used to detect the expression of Ezrin and FAK genes. Using RNA interference, intracellular expression of these two genes was suppressed. Moreover, flow cytometry was used to detect the cell anoikis. Compared to DMSO group, sclareol-treated cells had lowered expression of Ezrin and FAK genes, with the mRNA level decreased by 45% and 42%, respectively. The ratio of cell apoptosis was significantly elevated (P<0.05). RNA interference assay showed the enhancement of cell apoptosis by inhibiting Ezrin or FAK gene expression. Sclareol may promote the anoikis of MG63 osteosarcoma cells by inhibiting Ezrin and FAK gene expression.

Keywords: Sclareol, Ezrin, FAK, osteosarcoma, anoikis

Introduction

Osteosarcoma is one common primary malignant tumor in bone tissues with high incidence and mortality. It is most frequently occurred in children and young people below 20 years old [1]. With high malignancy and frequent invasiveness, tumor cells of osteosarcoma are predisposed to have blood borne metastasis toward brain, lung, kidney or liver [2]. Such distal metastasis significantly suppressed the cure rate of osteosarcoma, and is the major reason for tumor-related death, thus causing heavy burdens for patients and families [3]. Tumor metastasis includes various biological processes such as tumor migration and anoikis-resistance. The study of molecular mechanisms underlying metastasis of osteosarcoma may provide new insights for the diagnosis and treatment of osteosarcoma [4]. Anoikis is one

type of programmed cell death that is induced by the detachment of cells from adjacent cells [5]. As one special type of apoptosis, anoikis plays important roles in body development, pathogenesis and tumor metastasis [6]. Tumor cells own resistance against anoikis that prevents cells from death after the detachment from extracellular matrix (ECM). This function is of critical role in preventing tumor cell apoptosis after detachment from matrix and further blood transmission toward distal organ/tissue for further proliferation [7]. Further studies regarding the molecular mechanism of anoikis is pivotal for preventing and treating tumor metastasis. Previous findings have established important roles of Ezrin and focal adhesion kinase (FAK) in cellular signal transduction, cell cycle modulation and cell migration, and thus plays important roles in progression and metastasis of tumor cells [8]. Sclareol is one com-

pound of songarian sunrose alkane, and was initially isolated from sage clary for synthesizing drugs or cosmetics due to its lower toxicity [9]. Previous study has reported the anti-tumor effect of sclareol against the growth of leukemia, gastric carcinoma, colorectal cancer and osteosarcoma [10]. Sclareol has been suggested to inhibit tumor cell growth via facilitating cell apoptosis [11]. The mechanism for facilitating tumor cell apoptosis, however, remained unclear yet. This study treated MG63 osteosarcoma cells with poly HEMA to make cell suspensions. After sclareol treatment, both anoikis condition and gene expression of Ezrin and FAK genes were observed, in order to investigate the molecular mechanism of sclareol on regulating anoikis resistance of osteosarcoma cells.

Materials and methods

Reagents and equipment

DMEM culture medium, fetal bovine serum (FBS) and trypsin were produced by Gibco (US). DMSO and sclareol were purchased from Sigma (US). CCK8 test kit, reverse transcription kit and SYBR green fluorescent dyes were obtained from Toyobo Life Science (Japan). Annexin V-FITC/PI apoptotic test kit was produced by Yeasen (US). FAK antibody was produced by Abcam (US). Ezrin antibody and anti-rabbit antibody were obtained from Santa Cruz (US). CO_2 incubation chamber was a product of Thermo (US). Real-time PCR cycler model VIIA7 was produced by ABI (US). Flow cytometry was performed on equipment by BD Biosciences (US).

Cell line

MG63 osteosarcoma cells were purchased from Shanghai Institute, Chinese Academy of Sciences. Cells were incubated in DMEM medium containing 10% FBS and were kept in a humidified chamber with 5% CO_2 at 37°C.

Cell suspension culture

Poly HEMA was prepared in ethanol for 10 mg/mL dilution. 2 mL of poly HEMA working solutions were evenly applied onto 60 mm-culture dish. After one hour, 2 mL of poly HEMA solutions were applied again. The dish was dried for 24 hours in fume hood. MG63 osteosarcoma cells at log phase were digested in 0.25% trypsin for 3 min. Cells were then prepared for single-cell suspension and were inoculated in poly HEMA-precoated culture dish, with 3 mL DMEM medium. Cells were kept in a humidified chamber with 5% CO_2 at 37°C.

CCK8 assay

MG63 osteosarcoma cells in suspension culture were seeded into 96-well plate at 0.5×10^4 cells per well, and were incubated in CO_2 chamber. Cells were further divided into blank control, DMSO and sclareol groups. The gradient concentration of sclareol including c was used (N=6 each) for 48-hour incubation. After treatment, medium in wells was removed followed by PBS rinsing for 3 times. 0.1 mL 10% CCK8 solution was added for 4-hour incubation at 37°C. Absorbance values in each well were detected under a microplate reader at 450 nm. Using sclareol concentration as the horizontal axis, a growth curve for MG63 cells was plotted using survival rate as the vertical axis. The LC_{50} value of sclareol on suspension cultured MG63 cells within 48 hours was deduced.

siRNA transfection

Small interference RNA (siRNA) plasmids were generated using pUC18 plasmid as the vector. SiRNA sequence for Ezrin gene (Sense, 5-CTGGT CAAAT GCACT GTCC-3; Anti-sense, 5'-GCATG TGGCC TGCTA TGGA-3') and for FAK gene (Sense, 5'-GCATG TGGCC TGCTA TGGA; anti-sense, 5'-TCCAT AGCAG GCCAC ATGC-3'). Scramble siRNA was also applied as the control group. Plasmid vector can be used to synthesize viral particles within 24-hour incubation. Supernatants were collected and filtered in 0.45 μm filtering membrane. Those supernatants after filtration were re-added into MG63 osteosarcoma cells and were incubate din a humidified chamber with 5% CO_2 at 37°C. Six hours later, culture medium was changed for fresh DMEM medium.

Real time florescent quantitative PCR

Total RNA was extracted by Trizol method and was used to synthesize cDNA following manual instruction of reverse transcription kit. cDNA were then obtained and mixed with specific primers (95°C, 1 μL each), and PCR primers (see **Table 1**), 2XSYBR PCR Mixture and sterilized water. PCR conditions were 95°C for 5 min, followed by 40 cycles each containing 95°C for 15 sec, 60°C for 15 min and 72°C 45°C the whole mixture was detected on VIIA7.

Table 1. Specific primers for genotyping

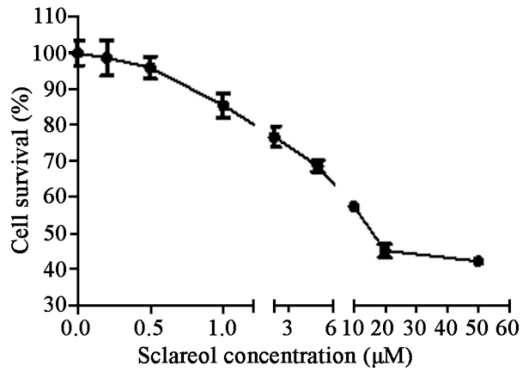| Gene	Forward primer	Reverse primer
β-actin	GAGGGAAATCGTGCGTGAC	CTGGAAGGTGGACAGTGAG
Ezrin	CCTCTCGAGAAGGATGGCGCAC	CTCTCGAGAAGGATGGCGCAC
FAK	GGCCATCGCAGGAGGCAGGA	AAGCTATCGCAGCACATCTCA

Figure 1. Effect of sclareol on proliferation of MG63 osteosarcoma cells.

Western blotting

MG63 cells were collected and mixed with RIPA lysis buffer including proteinase inhibitor at 4°C. Cell lysate was then prepared afterwards. After 30-min incubation, the protein concentration was determined by BSA method and was adjusted for 2 μg/μL. 30 μL protein samples were separated by 8% PAGE, and were transferred to NC membrane using 300 mA electrical field. The membrane was firstly blocked in 5% defatted milk powder, primary and secondary antibody were visualize onto pictures. Primary antibody (1:1000) and secondary (1:1000 to 10:0000) were sequentially added for 4°C overnight incubation and room temperature. GIS-2020D software was used to analyze the light intensity of protein bands for estimating expression level of each protein.

Flow cytometry

Cells were collected and centrifuged at 1000 g for 5 min. The supernatant was removed, followed by the addition of PBS, followed by further 1000 g for 5 min. The supernatant was against removed and mixed with blocking buffer. After room temperature incubation for 10 min, the mixture was centrifuged for 5 min to remove supernatants and added with 0.1 mL Annexin V labelled solution for further 10-min incubation. In the end, cells were centrifuged,

with supernatants removed and addition of PI dye. After 10-min incubation, the sample was analyzed in a flow cytometry.

Transwell cell invasion assay

MG63 osteosarcoma cells were collected and were divided into 6 groups, namely DMSO, sclareol, Ezrin RNAi, siNC control, FAK RNAi and siNC control. All cells were incubated for 48 hours until removing culture medium. Cells were then collected by centrifugation and were mixed with serum-free DMEM medium for 24-hour incubation. Cells were then collected by centrifugation and were seeded into plate containing 2×10^4 density. After 24 hours, those cells on the surface of membrane were firstly fixed in paraformaldehyde and were counter-stained by hematoxylin. An inverted microscope was used to observe the morphology of our samples.

Statistical analysis

SPSS 16.0 software is used to perform major analysis of experiment data. Enumeration data were presented as % and were tested by chi-square analysis. Measurement data was presented as mean ± standard deviation (SD) and were compared by analysis of variance. A statistical significance was defined when $P < 0.05$.

Results

Cytotoxicity of sclareol on MG63 cells

We firstly used CCK8 assay for comparing the cell survival between blank control, DMSO and gradient sclareol (0.2 μM, 0.5 μM, 1.0 μM, 2.0 μM, 5.0 μM, 10.0 μM, 20.0 μM and 50.0 μM, N=6 each). After 48 hours of incubation, CCK8 test kit was applied for measuring cell proliferation. As shown in **Figure 1**, sclareol has certain cytotoxicity on MG63 osteosarcoma cells. With higher concentration of sclareol, the survival percentage of cells was gradually decreased (**Figure 1**). By calculation, LC_{50} of sclareol on MG63 cells was about 14 μM at 48 hour. These data suggested the cytotoxicity of sclareol on MG63 cells and suppressed cell proliferation.

Effect of sclareol on suspension cultured MG63 cells' apoptosis

CCK8 assay revealed the suppressed growth of MG63 cells by sclareol. Based on LC_{50} values,
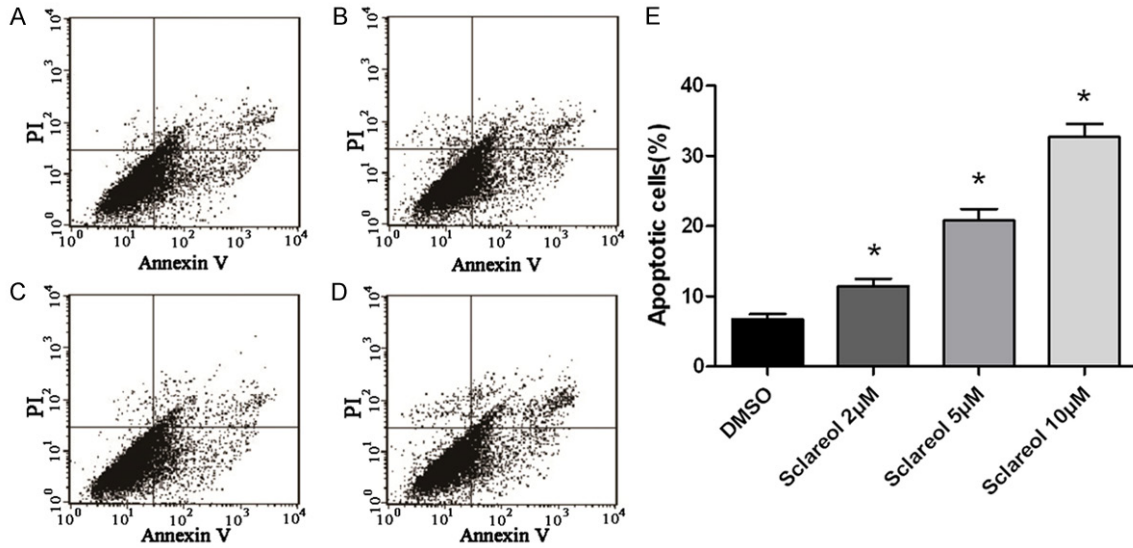
Figure 2. Induction of MG63 cell apoptosis by sclareol. A. MG63 apoptosis in DMSO group; B. 2.0 µM sclareol group; C. 5.0 µM sclareol group; D. 10.0 µM sclareol group; E. Percentage of apoptotic cells induced by different concentrations of sclareol. *P<0.05 compared to DMSO group.
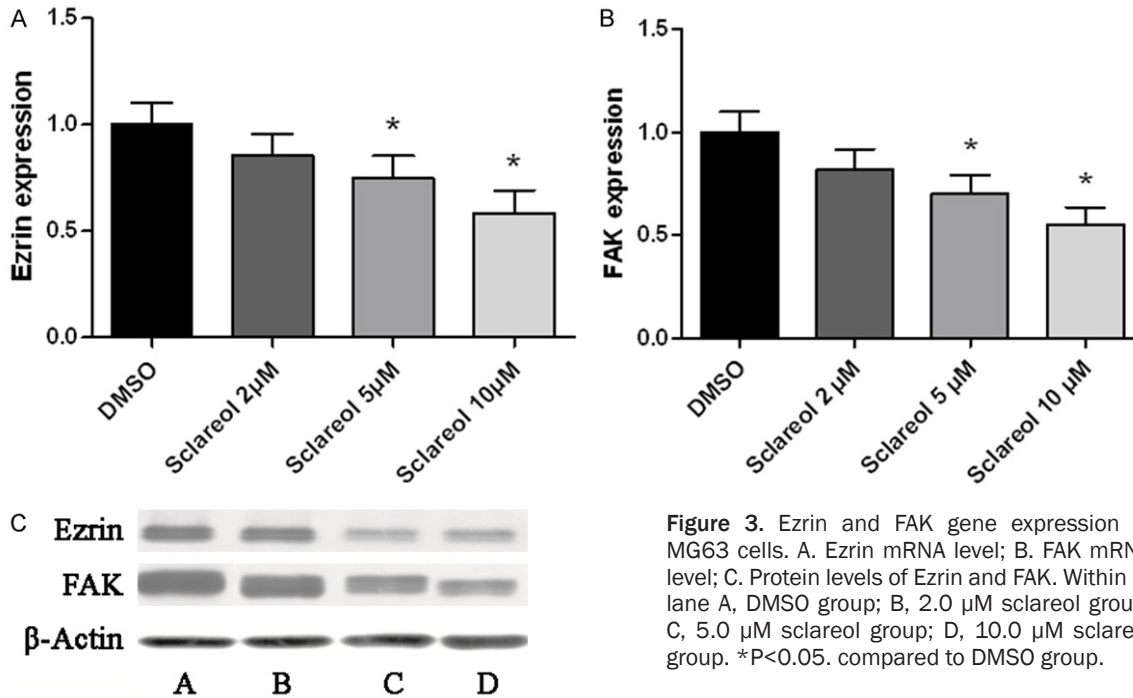

Figure 3. Ezrin and FAK gene expression in MG63 cells. A. Ezrin mRNA level; B. FAK mRNA level; C. Protein levels of Ezrin and FAK. Within C, lane A, DMSO group; B, 2.0 µM sclareol group; C, 5.0 µM sclareol group; D, 10.0 µM sclareol group. *P<0.05. compared to DMSO group.

we selected 2.0 µM, 5.0 µM and 10.0 µM to test the apoptosis of MG63 cells using flow cytometry. As shown in **Figure 2**, compared to DMSO group, the apoptotic percentage of MG63 osteosarcoma cells in suspension culture was significantly elevated (11.2%, 20.8% and 32.5% vs. 6.7%, P<0.05), suggesting the facilitation of cell growth by sclareol.

Effects of sclareol on Ezrin and FAK gene expression

Abovementioned experiments reported that sclareol might facilitate cell apoptosis and inhibit cell proliferation. Based on previous reports, we detect Ezrin and FAK gene expression under DMSO and (low, moderate and high
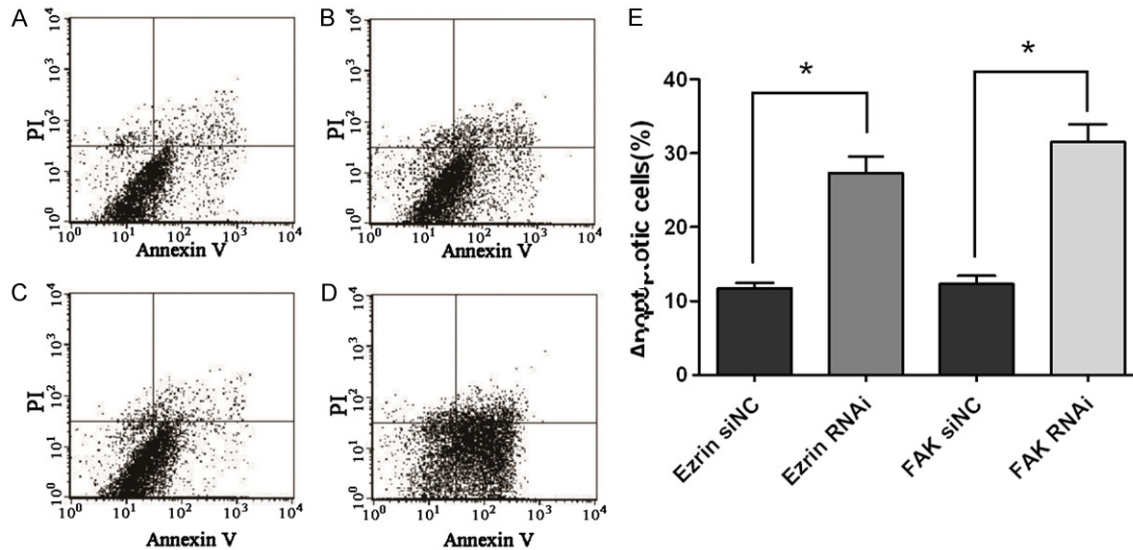
Figure 4. MG63 cell apoptosis by Ezrin and FK gene modulation. A. Ezrin-siNC group; B. Ezrin RNAi group; C. FAK siNC group; D. FAK RNAi group; E. Percentage of MG63 cell apoptosis after transfection of siRNA against Ezrin or FAK. *P<0.05 compared to siNC group.

dosage) sclareol groups. Both Ezrin and FAK genes had significant decreased expression compared to DMSO (33% for Ezrin and 36% for FAK, P<0.05, **Figure 3A** and **3B**). Western blotting assay also showed significantly lowered expression of Ezrin and FAK genes in sclareol-treated groups compared to DMSO group (**Figure 3C**). The expression of these two genes was negatively related with sclareol dosage, clearly suggesting the inhibition of Ezrin and FAK gene in MG63 cells by sclareol.

Cell apoptosis after Ezrin and FAK gene silencing

We further suppressed Ezrin and FAK gene expression in osteosarcoma cells, whose apoptotic ratio was observed by flow cytometry. As shown in **Figure 4**, compared to siNC control ones, apoptotic cell ratio was significantly higher in treatment group (P<0.05). Among those cells, the apoptotic percentage was 27.8% (**Figure 4B**) and 31.5% (**Figure 4D**) in Ezrin-RNAi and FAK-RNAi cells. These results suggested the facilitation of cell death by down-regulating Ezrin and FAK expression.

Effects of Ezrin and FAK gene on MG63 cell invasion

To further illustrate the role of Ezrin and PAK gene in migration of tumor cells, we observed the invasion by Transwell assay after inhibiting

the expression of Ezrin and FAK genes. As shown in **Figure 5**, Ezrin gene silencing caused significantly lowered number of invasion cells as compared to control group (82.6±9.2 vs. 162.5±11.7, **Figure 5A** and **5B**). FAK gene silencing also decreased invasion cell number (76.1±8.9 vs. 149.2±13.1, **Figure 5C** and **5D**). These data suggested significant suppression of tumor's invasion ability by inhibiting Ezrin and FAK gene expression.

We further explored the effect of sclareol on MG63 cell invasion by treating cells with DMSO or gradient concentrations of sclareol (2 μM, 5 μM and 10 μM). As shown in **Figure 6**, DMSO treated cells had 151.4±14.7 invasion cells (**Figure 6A**), while sclareol treated cells had 125.8±12.1, 108.2±10.7 and 72.9±8.1 for 2 μM, 5 μM and 10 μM, respectively (**Figure 6A-D**). These results indicated the inhibition of sclareol on invasion of MG63 cells.

Discussion

The coherent function of body organs which consist of various cells largely depends on the interconnection of cells [12]. Such cell-to-cell connection is formed by the extracellular matrix, lack of which can cause cell death called anoikis [13]. As one special type of apoptosis, anoikis plays an important role in body growth, pathogenesis and tumor metastasis. Some cells, however, may not undergo anoikis
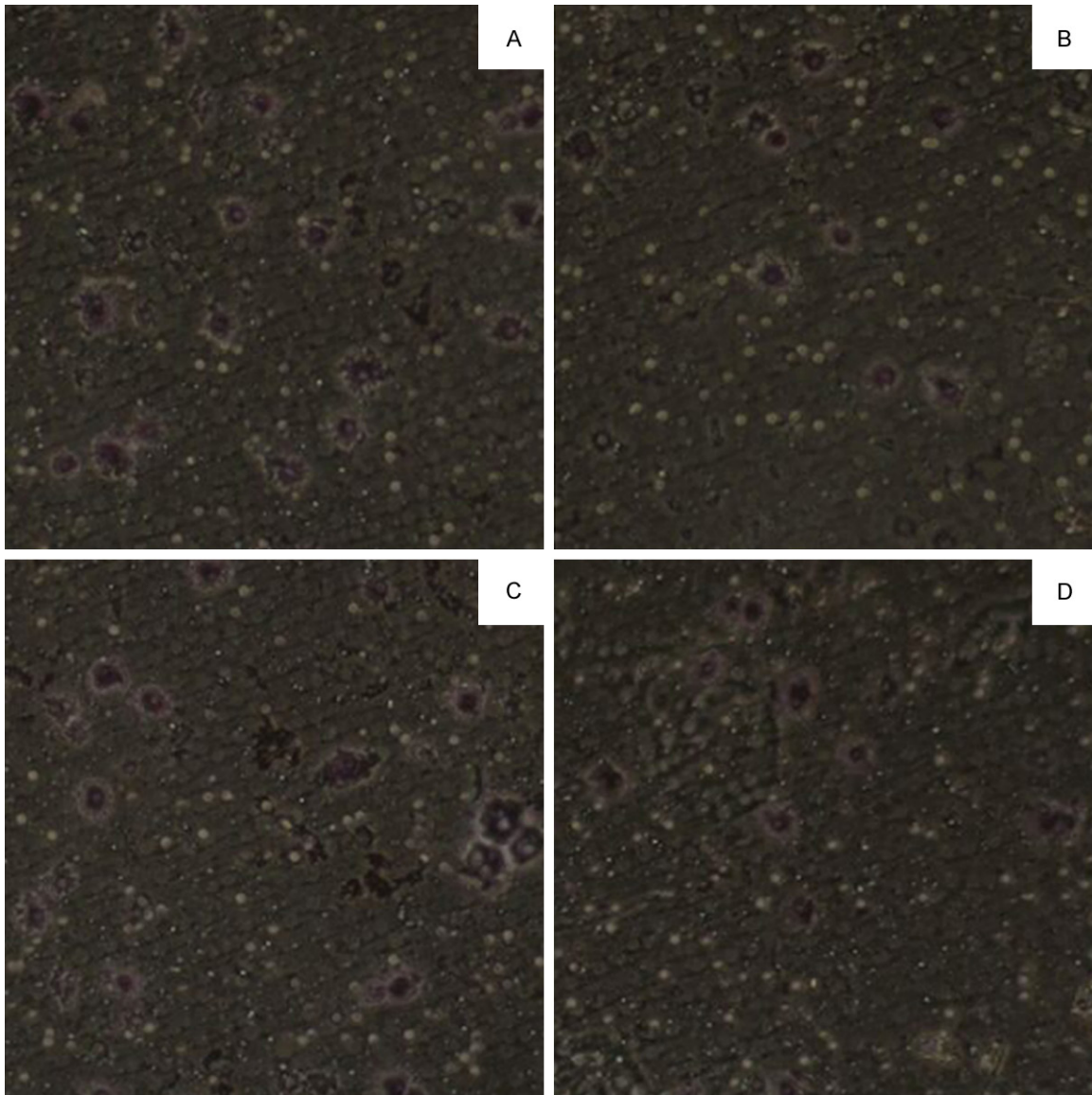
Figure 5. Invasion of MG63 cells. A. Ezrin-siNC group; B. Ezrin RNAi group; C. FAK siNC group; D. FAK RNAi group.

even after detaching from extracellular matrix, and can have distal metastasis, indicating the anoikis resistance of those cells [14]. Recent study has shown the critical role of anoikis resistance in tumor metastasis [15]. As one complicated biological process, tumor metastasis requires the acquirement of anoikis resistance of tumor cells [16]. Therefore, the study of mechanism underlying anoikis resistance of tumor cells, it is of critical importance for studying the mechanism of tumor metastasis.

Sclareol is one compound extracted from sage clary for synthesizing drugs or cosmetics due to its low toxicity. Recent study, however, suggest-

ed the anti-tumor activity of sclareol in osteosarcoma, breast cancer and colorectal carcinoma [17]. Therefore this study focused on the effect of sclareol on anoikis resistance of MG63 osteosarcoma cells, in addition to the possible molecular mechanism. We treated MG63 cells with poly HEMA to make it suspension. Previous study has reported the detachment of single suspended cells with anoikis due to the absence of extracellular matrix [18]. CCK8 study has shown the toxicity of sclareol on MG63 cells as the retard of cell growth and proliferation. Flow cytometry results showed significantly elevated apoptotic cell percentage after MG63 treatment.
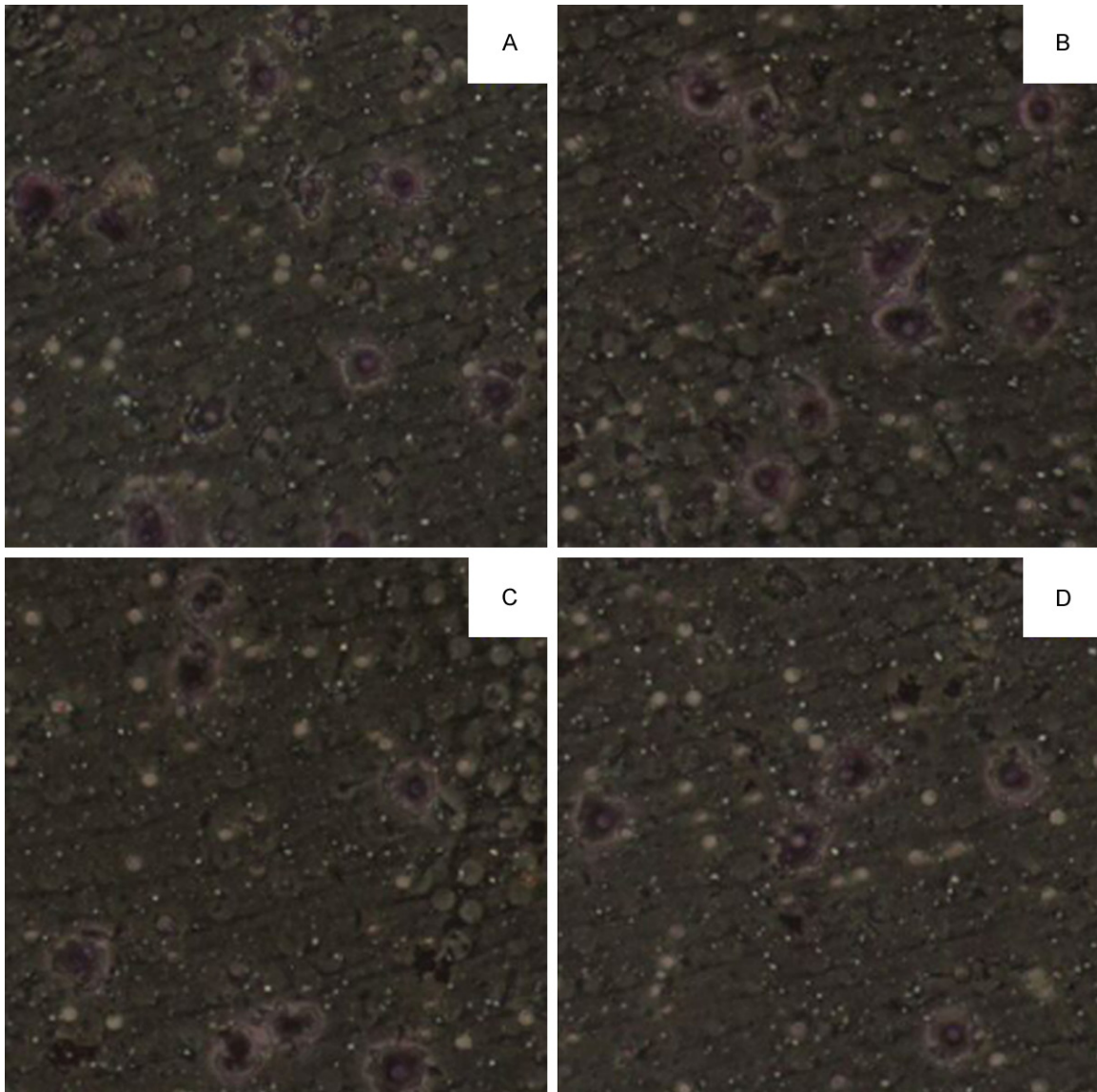
Figure 6. Effect of sclareol on MG63 osteosarcoma cell invasion. A. Ezrin-siNC group; B. Ezrin RNAi group; C. FAK siNC group; D. FAK RNAi group.

The mechanism of sclareol on anoikis of tumor cells remains unknown yet. Previous findings suggested the possible involvement of Ezrin protein and FAK-induced signaling pathway [19]. As one member of ERM protein family, Ezrin is one surface receptor for intercellular connection. Study has found the critical role of Ezrin in the interaction between cells, and between cells and matrix, in addition to the tumor metastasis and invasion [20]. FAK is one type of cytoplasmic non-receptor type of tyrosine kinase and belongs to tyrosine kinase superfamily. Study has suggested the participation of FAK in embryonic development, prolif-

eration, tumor migration and invasion as one critical molecule in cellular signaling transduction [21]. Based on this, we examined the expression of Ezrin and FAK genes in sclareol treated MG63 cells and found lowered expression of these two genes in experimental group, suggesting the inhibition of Ezrin and FAK gene expression by sclareol. Further studies revealed the facilitation of MG63 cell apoptosis by decreasing Ezrin and FAK gene expression, which is consistent with previous findings [22, 23]. These results suggested the inhibition of Ezrin and FAK gene expression in MG63 cells that lead to anoikis. Previous findings also

showed the relationship between anoikis resistance and tumor migration and invasion abilities [24]. To further study the mechanism of sclareol on osteosarcoma cell anoikis resistance, we suppressed Ezrin and FAK gene expression to observe the invasion of cells. Transwell assay found lower invasion ability of MG63 cells after sclareol exposure. Meanwhile, the suppressing of Ezrin and FAK gene expression also impaired cell invasion, suggesting the possible role of sclareol in decreasing MG63 cell invasion by inhibiting Ezrin and FAK gene expression.

In summary, sclareol had certain cytotoxicity on MG63 osteosarcoma cells as it inhibited cell proliferation. It can also enhance the anoikis resistance and decreased cell invasion of osteosarcoma cells via suppressing intracellular expression of Ezrin and FAK genes. Sclareol thus has potential roles for inhibiting osteosarcoma cell proliferation or tumor metastasis.

Acknowledgements

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jianwen Mo, Department of Orthopedics Surgery, The First Affiliated Hospital of Gannan Medical University, 23 Qingnian Road, Ganzhou 341000, Jiangxi, China. Tel: +86-797-8283927; Fax: +86-797-8283927; E-mail: jianwenmoly@163.com

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