Am J Transl Res 2019;11(4):2117-2139
www.ajtr.org /ISSN:1943-8141/AJTR0092183

Original Article

High expression of ID1 facilitates metastasis in human osteosarcoma by regulating the sensitivity of anoikis via PI3K/AKT depended suppression of the intrinsic apoptotic signaling pathway

Guo-Sheng Zhao[1,3], Qiao Zhang[4], Ya Cao[2], Yang Wang[1], Yang-Fan Lv[2], Zhao-Si Zhang[5], Yuan Zhang[6], Qiu-Lin Tan[2], Yu Chang[1], Zheng-Xue Quan[1], Dian-Ming Jiang[1,3], Qiao-Nan Guo[2]

[1]Department of Orthopedic Surgery, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, People's Republic of China; [2]Department of Pathology, Xinqiao Hospital, Army Medical University, Chongqing 400037, People's Republic of China; [3]Bone and Trauma Center, The Third Affiliated Hospital of Chongqing Medical University, Chongqing 401120, People's Republic of China; [4]Department of Rehabilitation, Xinqiao Hospital, Army Medical University, Chongqing 400016, People's Republic of China; [5]Department of Neurosurgery, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, People's Republic of China; [6]Department of Orthopaedics, Children's Hospital of Chongqing Medical University, Ministry of Education Key Laboratory of Child Development and Disorders, Key Laboratory of Pediatrics in Chongqing, China International Science and Technology Cooperation Base of Child Development and Critical Disorders, Chongqing 400014, People's Republic of China

Received February 2, 2019; Accepted February 21, 2019; Epub April 15, 2019; Published April 30, 2019

Abstract: A lack of understanding of the molecular basis underlying the regulation of metastatic disease and its effective therapy are the primary causes of high mortality in osteosarcoma. Thus, new insights into metastases and novel effective targets for metastatic osteosarcoma are urgently required. Anoikis resistance is considered a hallmark of cancer cells with metastatic ability. However, the molecular mechanism of anoikis is poorly understood in osteosarcoma. We applied immunohistochemistry to investigate the correlation between inhibitor of differentiation or DNA binding 1 (ID1) and clinicopathological features, and investigated the correlation between ID1 and the metastatic behavior of osteosarcoma cells, in vitro and in vivo. The results revealed that ID1 is overexpressed in human osteosarcoma tissues, is positively associated with lung metastases, and is a potential biomarker of poor prognosis. Overexpression of ID1 could increase anoikis insensitivity of osteosarcoma cells to facilitate metastasis through the PI3K/AKT-dependent mitochondrial apoptosis pathway. Knockdown of ID1 partly reversed the high potential of metastasis in anoikis-resistant osteosarcoma cells. Our findings revealed, that ID1 is a candidate molecular target for metastatic potential osteosarcoma by highlighting the role of anoikis resistance. In addition ID1 might be a potential predictor of poor prognosis in patients with osteosarcoma.

Keywords: Osteosarcoma, anoikis, inhibitor of differentiation or DNA binding 1, metastasis, biomarker

Introduction

Osteosarcoma is the most frequent primary pediatric malignancy of bone and is also a common cause of cancer-related death in children [1]. Since multiagent chemotherapies combined with surgery became the first-line treatment for osteosarcoma twenty years ago, the five-year survival rate has increased to 64% in children [2]. However, over the last two decades, the treatment of osteosarcoma has not dramatically improved and the five-year survival rate remains approximately 65-70% [3]. In contrast to localized disease, the effective therapy for patients with metastatic osteosarcoma has not been fully established, resulting in the five-year survival rate for metastatic disease remaining around 20% [4-6]. Thus, novel targets and therapies for osteosarcoma, especially to inhibit metastasis of osteosarcoma, are urgently required.

Similar to other types of cancer cells, metastasis of osteosarcoma cells results from a com-

plex series of procedures, including cell migration and invasion; detachment from the extracellular matrix (ECM); entry into the circulation; and finally, metastatic colonization at the distant organs [7]. However, most of the cancer cells undergo apoptosis and die when they are detached from the ECM or during circulation. This special type of apoptotic cell death triggered by a lack of survival signals generated from the ECM and neighboring cells is called anoikis [8, 9]. Anoikis is important to prevent normal cells from surviving in circulation and growing in the wrong sites. Meanwhile, it provides a barrier to cancer metastasis [10]. Therefore, the anoikis resistance of cancer cells is presumed to play a key role in metastatic behavior. In addition, there is increasing evidence that resistance to anoikis facilitates metastasis in osteosarcoma [9-11], suggesting that restoration of anoikis sensitivity may be an effective means to inhibit metastasis. Several studies, including our previous works, have provided some insight into how osteosarcoma develops anoikis resistance, such as transcription factors, oncogenes, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/AKT kinase (AKT) or mitogen activated protein kinase (MAPK) pathway activation, and interactions between bone marrow-derived mesenchymal stem cells [9-14]. Moreover, anoikis-resistant subpopulations of osteosarcoma cells displayed significant angiogenesis and chemoresistance during circulation [12, 15]. Nevertheless, little is known about the genes that control this process, and the molecular mechanisms underlying metastasis and resistance to anoikis in osteosarcoma remain incompletely understood.

Inhibitor of differentiation or DNA binding 1 (ID1), one of the helix-loop-helix (HLH) family of proteins, has a crucial role during normal development, malignant transformation, and cancer progression [16]. ID1 has been recognized as a tumor promoter in several types of malignant tumors, such as colon cancer, thyroid cancer, gastric cancer, and hepatocellular carcinoma [16-18]. Moreover, high expression of ID1 is also believed to facilitate cancer metastasis, making it a potential candidate anti-cancer metastasis target [17]. Importantly, ID1 is involved in bone formation by regulating the osteoblastic differentiation of mesenchymal stem cells [18]. There may be a close connection between dysregulation of osteoblast differentiation and tumorigenesis of primary bone tumors [19]; therefore, ID1 may be an effective molecular target for osteosarcoma. However, little is known about the relationship between ID1 and osteosarcoma progression [20, 21]. However, ID1 gene expression is significantly upregulated in osteosarcoma tissues compared with that in non-malignant bone tissues [20] and overexpression of ID1 promotes human osteosarcoma cell growth and resistance to apoptosis through activation of the PI3K/AKT pathway [21]. Despite these limited findings, the role and regulation of ID1 in the metastatic behavior of osteosarcoma remains unknown.

In the present study, we investigated the association between ID1 expression and the clinicopathological features in tissues of patients with osteosarcoma. We found that higher expression of ID1 correlated with lung metastasis and poor prognosis, and promoted metastasis by preventing anoikis, rather than facilitating cell migration and invasion. Moreover, knockdown of ID1 reversed the acquired anoikis resistance of osteosarcoma cells. In addition, the PI3K/AKT-mediated intrinsic apoptotic (mitochondrial) signaling pathway was found to be involved in ID1-induced inhibition of anoikis. To the best of our knowledge, no previous study has demonstrated the role of ID1 in metastasis and anoikis resistance in osteosarcoma. The results demonstrated that ID1 could be a new molecular target of osteosarcoma. We provided novel insights into the role and the underlying mechanism by which ID1 promotes metastasis in osteosarcoma by highlighting the role of anoikis resistance.

Materials and methods

Human specimens

Specimens were obtained from 67 patients with histopathologically confirmed osteosarcoma with no preoperative anticancer treatment from the Southwest Hospital and Xinqiao Hospital of Army Medical University (AMU), Chongqing, China, from February 2011 to October 2017. Local recurrence and distant metastasis were diagnosed using imaging and pathology. The tumor stages were classified according to the Enneking system. Patients with primary

Table 1. Primers used for qRT-PCR in this study

Target gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
ID1	ACGGCTGTTACTCACGCCTC	GCTGGAGAATCTCCACCTTGC
MMP2	CCCACTGCGGTTTTCTCGAAT	CAAAGGGGTATCCATCGCCAT

The primers were all obtained from Sangon Biotech (Sangon, Shanghai, China).

osteosarcoma were classified as with or without developed distant metastasis at diagnosis or after surgery. The control group of histopathologically-confirmed benign tumor specimens were obtained from 21 patients with fibrous dysplasia and 12 patients with osteoblastoma from Xinqiao Hospital from 2015 to 2017. Written informed consent for these experimental studies was obtained from the patients or their guardians. All experiments were approved by Southwest Hospital (No. 27-2011) and Xinqiao Hospital (No. 2018-069-01) ethics committees.

Cell culture and compounds

The human osteosarcoma cell lines 143B and MNNG/HOS (M/HOS) were obtained from Cellcook Biological Technology Co., Ltd (Guangzhou China). Cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, BI, Kibbutz Beit Haemek, Israel) and 1% penicillin-streptomycin (Hyclone). All the cells were cultured at 37°C in 5% CO_2 and a humidified atmosphere.

Cells were treated with the PI3K/AKT inhibitor LY294002 (25 μM, Selleck Chemicals, Houston, TX, USA) for 24 h, as required.

Lentivirus transfection

The HBLV-h-Puro expressing lentiviral vector for ID1 and the control were synthesized and obtained from Hanbio Biotechnology Co., Ltd (Hanbio, Shanghai, China) and the reference sequence for human ID1 was acquired from NCBI (NM_002165.3). The short hairpin RNA (shRNA) in the pHBLV-U6-ZSGreen-puro lentiviral vector targeting ID1 and its scrambled control shRNA were also purchased from Hanbio. 143B, M/HOS, and 143B-AR cells (anoikis-resistant 143B cells, produced in this study) were infected with the indicated lentivirus vectors according to the manufacturer's instructions, as previously described [6]. The shRNA target sequences are "TTCTCCGAACGTGTCACGTAA" for scrambled and "GCAAGAACTGCTTCGGCAGGTCCTC" for shID1. The overexpression or knockdown function was verified by quantitative real-time PCR and western blotting analysis.

Transfection of short interfering RNAs

Short interfering RNAs (siRNAs) targeting ID1 were used for transient knockdown experiments and were purchased from Ribobio Biotechnology Co., Ltd (Ribobio, Guangzhou, China). Cells were transfected with 20 nM targeting siRNA (two sequences) or scrambled siRNA using ribobio-FECT™ CP (Ribobio) according to the manufacturer's instructions. Cells were assayed 24-48 h after transfection. Knockdown efficiency was assessed using quantitative real-time PCR (qPCR) and western blotting analysis. The siRNA target sequences used in this experiments are "GAACTCGGAATCCGAAGTT" for ID1-siRNA-1, "CACGTCATCGACTACATCA" for ID1-siRNA-2 and "TCAGGGACCTTCAGTTGGA" for ID1-siRNA-3.

Quantitative real-time PCR analysis and western blotting analysis

Quantitative real-time PCR (qPCR) was performed as described previously [22]. The details of the primers are listed in **Table 1**.

For western blotting analysis, proteins were separated using 10-12% SDS Tris-glycine gels and transferred to a polyvinylidene fluoride (PVDF) membrane (3010040001, Roche, Shanghai, China). The membrane it was blocked with 5% fat-free milk and incubated with the indicated primary antibodies overnight at 4°C. Antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000, AB-P-R001) were purchased from Hangzhou Goodhere Biotechnology Co., Ltd (Hangzhou China). Antibodies against matrix metalloproteinase 2 (MMP2) (1:500, 40994S), phosphorylated AKT kinase (p-AKT) (1:1000, 4060S), cleaved caspase 3 (1:500, 9664T), B-cell CLL/lymphoma 2 (BCL2) (1:1000, 2872T), cytochrome c (1:1000, 11940T), and BCL2 associated X protein (BAX)

(1:500, 5023T) were purchased from Cell Signaling Technology (CST, Danvers, MA, USA); the ID1 antibody (1:500, bs-6541R) was purchased from Bioss (Beijing, China). Antibodies against extracellular signal-regulated kinase (ERK) (1:1000, AF1051), p-ERK (1:1000, AF1891), and AKT (1:1000, AF1789) were purchased from Beyotime Biotechnology (Beyotime, Shanghai, China). After incubation with the primary antibodies, the secondary antibody (goat anti-rabbit, Bioss) was applied. Immunoreactivity was detected using an ECL Kit (BeyoECL Moon, Beyotime).

Anoikis analysis

Cell anoikis (apoptosis) was analyzed using Annexin V-phycoerythrin (PE)/7-aminoactinomycin D (7-ADD) analysis using an Annexin V-PE/7-ADD kit (Becton Dickinson, BD, Franklin Lakes, NJ, USA) according to the manufacturer's protocols. After the cells were infected with the ID1 overexpression vector, or siRNAs targeting ID1, the cells were seeded in ultra-low attachment 6-well plates (Corning, NY, USA) and cultured for 2 days. The indicated inhibitors were added into the plates for the last 24 hours if required. Thereafter, the cells were harvested and washed with phosphate-buffered saline (PBS), and then resuspended in 200 μl of binding buffer. Subsequently, 5 μl of Annexin V-PE and 5 μl of 7-ADD were added and incubated for 15 min at room temperature in the dark. Flow cytometric analysis was then performed using a flow cytometer (FACSCalibur, BD, USA). Annexin V-PE positive/7-ADD negative cells were identified as early apoptotic cells. Annexin V-PE and 7-ADD positive cells were identified as late apoptotic cells.

Anoikis-resistant cell model

To establish the anoikis-resistant model, 5×10^5 cells were cultured in ultra-low attachment 6-well plates with DMEM supplemented with 10% FBS for 7 days as suspension cultures, then harvested, digested to a single-cell suspension, and transferred to attachment 24-well plates with normal culture until they formed monolayers. Then, 5×10^5 cells were cultured in suspension again for another 14 days, and finally transferred to attachment plates [8, 12]. The re-adherent cells were cultured in adhered conditions. The anoikis-resistant ability of the re-adherent cells were assessed by anoikis analysis as section 2.6. Cells were considered anoikis-resistant (-AR) when the anoikis was significantly decreased compared with that in untreated cells [8, 12]. The cellular morphology was observed under an inverted microscope (Olympus, Tokyo, Japan).

Mitochondrial membrane potential analysis

Changes in mitochondrial transmembrane potential occurring during anoikis induced by siRNAs targeting ID1 were examined using a mitochondrial membrane potential assay kit with JC-1 (C2006, Beyotime). As previously reported [23], after transfection, suspension cells were cultured in ultra-low attachment 6-well plates for 2 days; and then harvested and incubated with 1 ml JC-1 working solution, and incubated for 20 min at 37°C. Thereafter, the cells were washed with JC-1 staining buffer twice and analyzed by the FACSCalibur software (BD). JC-1 monomers or JC-1 positive cells were defined as having a loss of mitochondrial membrane potential, according to the negative (adhered with non-treatment cells) and positive controls (carbonyl cyanide m-chlorophenyl hydrazone (CCCP), 10 μM, 10 min).

R2 database analysis

The R2 database (http://hgserver1.amc.nl) was used to investigate the relationship between ID1 and other genes for patients with osteosarcoma. The data named mixed osteosarcoma-Kuijjer-127-vst-ilmnhwg6v2 was selected. In 'Select type of analysis' section, we selected 'correlate 2 genes' to analyze the correlation between ID1 and the common matrix metalloproteinases (MMPs) genes related to the invasive ability of osteosarcoma cells in 127 specimens using the Pearson rank correlation test [24].

Histology and immunohistochemistry

Immunohistochemistry (IHC) was performed using an IHC kit (Zsbio, Beijing, China). As reported previously, sample sections were deparafinized through a series of xylene baths, antigens were retrieved by steam treatment in 10 mM citrate buffer, blocked with 3% hydrogen peroxide for 15 min at 37°C, pre-incubated with blocking serum solution for 30 min at 37°C, and then incubated at 4°C with primary rabbit anti-human ID1 antibodies (1:150, bs-6541R, Bioss) overnight. Subsequently, the secondary antibodies were applied and the nuclei were counterstained with hematoxylin. Specimens were independently scored by two pathologists who were blinded to the clinical data. For the semi-quantitative assessment of

protein expression, the percentage of positive cells was calculated in no less than five randomly selected fields of view using higher-magnification objectives (× 400), and included over 50 cells. The final IHC score was a product of the positive cell ratio score (0 = no immunoreactivity; 1 ≤ 25% cells stained; 2 = 26-50% cells stained; 3 = 51-75% cells stained; and 4 = ≥ 76% cells stained) and the relative expression score (0, negative; 1, yellow staining; 2, brown staining; 3, dark brown staining) [6, 25]. Final scores ≥ 5 were considered high expression of ID1 (the average score was 4.97).

In vitro migration and invasion assays

Cells migration and invasion were assessed using wound-healing assays and Transwell invasion assays, respectively, as described previously [6]. Briefly, after the indicated treatments, cells were seeded into 6-well plates and a 10-μl micropipette tip was used to make scratches. Cells were monitored at 0 h and 20 h after scratching and images of wound healing were captured using a inverted phase contrast light microscope (Olympus) and DP Controller software (Olympus). Cellular migratory ability was quantified by measuring the wound healing index; i.e., the wound area healed by the cells at 20 h after scratching relative to the wound area at 0 h, using the ImageJ software. For the Transwell invasion assay, cells suspensions of 2×10^5 cells/ml in serum-free DMEM were seeded into the upper chambers, which were pre-coated in 1:3 diluted matrigel (BD) of 8-μm Transwell filters (Merck Millipore, Berlin, Germany), in a volume of 200 μl. DMEM containing 10% FBS was added to the lower chambers. Then the cells were incubated for 24 h. After 0.1% crystal violet staining for 5 min, the invaded cells were quantified in no less than four randomly selected fields.

In vivo lung metastasis model

The lung metastasis models were established as reported previously [6, 10]. Briefly, 25 female nude mice (Laboratory Animal Center, Xinqiao Hospital, AMU) were randomly divided into five groups as M/HOS-ctrl, M/Hos-lv ID1, 143B, 143B-AR Sh-Ctrl and 143B-AR Sh-ID1. To emphasize the influence of anoikis, with appropriate treatments, relative fewer cells (10^6) in a volume of 0.1 ml PBS were injected into the tail vein of each 5-week-old nude mouse. The mice were evaluated every four days for weight change and tumor burden with emaciation. The mice were sacrificed at 24 days after injection. The lungs of all the mice were resected, fixed in 10% formalin, and then embedded in paraffin, sectioned, and stained with H&E. Five discontinuous and deep sections were used to define whether there was a metastatic tumor nodule, and the number of lung metastasis nodules was counted in one section with the most nodules under a light microscope.

All the animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of Xinqiao Hospital, AMU, and were performed according to the Guide for the Care Use of Laboratory Animals.

Statistical analysis

Categorical data were analyzed using the Chi-squared test. Quantitative data were presented as the mean ± SD and analyzed using unpaired Student's t-tests for two groups and analysis of variance (ANOVA) with Bonferroni's multiple comparisons for more than two groups. Survival analysis (61 of the 67 patients) was carried out using the Kaplan-Meier method and log-rank test. All analyses were performed using SPSS 20.0 software (version 20.0, IBM Corp., Armonk, NY, USA) or GraphPad Prism (version 7.00, GraphPad Software, Inc., San Diego, CA, USA). All the in vitro experiments were performed at least in triplicate.

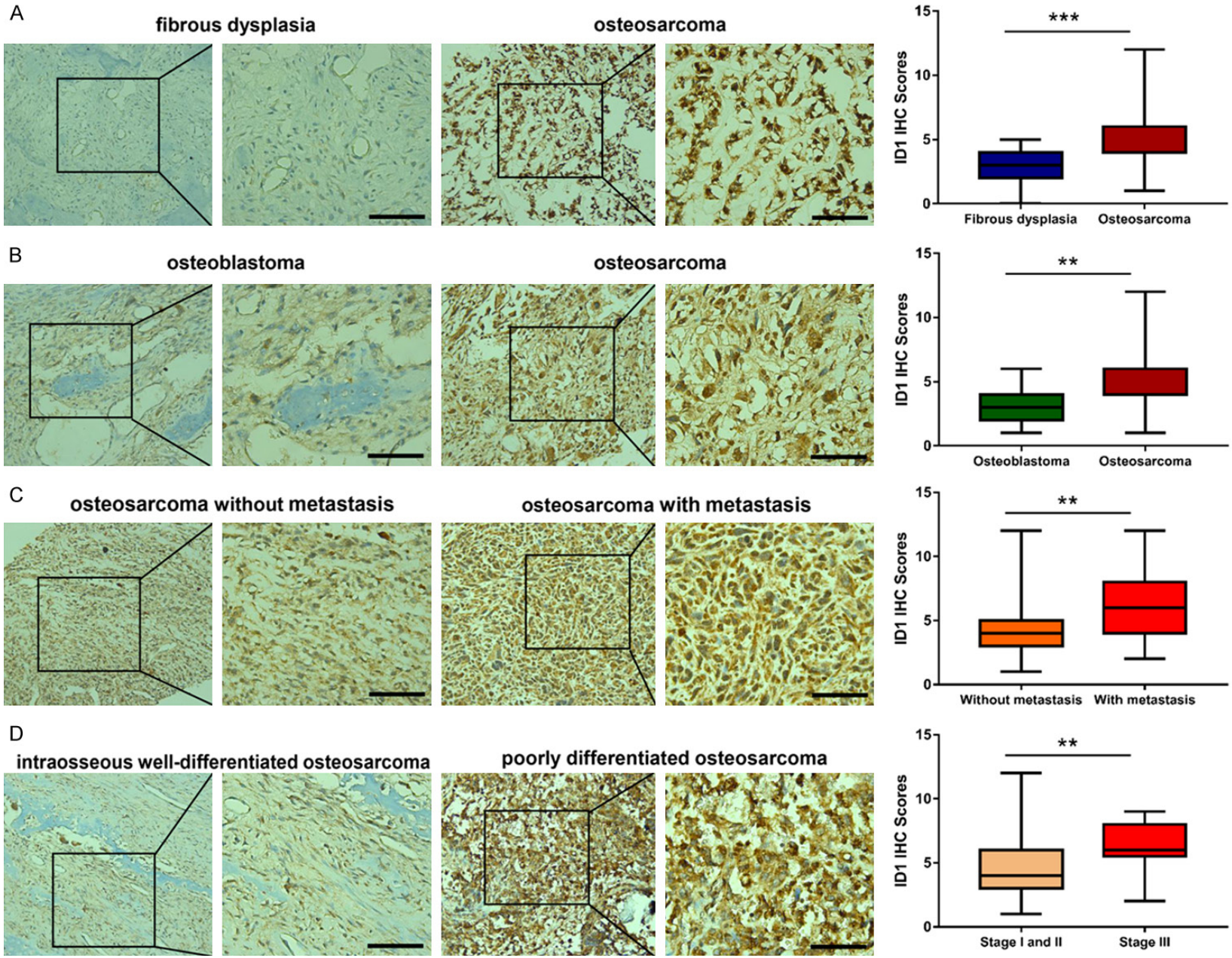
Results

ID1 is overexpressed in human osteosarcoma tissues

Previous studies have reported that the expression of ID1 is associated with progression in many types of malignant tumors [16-18, 26]. In addition, ID1 mRNA expression is increased in osteosarcoma tissues compared with that in corresponding normal tissues [20, 21]. To confirm this finding at the protein level, we applied IHC staining to examine ID1 expression between human benign bone tumors and osteosarcoma tissues. As expected, we observed significantly higher levels of ID1 ($P < 0.05$) in osteosarcoma compared with that in benign bone tumors (osteoblastoma or fibrous dysplasia) (**Figure 1A**, **1B**).

High expression of ID1, associated with distant metastasis and predicts a poor prognosis in human osteosarcoma

To examine the relationship between ID1 expression and osteosarcoma metastasis, we


A
fibrous dysplasia
osteosarcoma
ID1 IHC Scores
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Fibrous dysplasia
Osteosarcoma
B
osteoblastoma
osteosarcoma
ID1 IHC Scores
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Osteoblastoma
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C
osteosarcoma without metastasis
osteosarcoma with metastasis
ID1 IHC Scores
**
Without metastasis
With metastasis
D
intraosseous well-differentiated osteosarcoma
poorly differentiated osteosarcoma
ID1 IHC Scores
**
Stage I and II
Stage III

Figure 1. ID1 is overexpressed in osteosarcoma, positively associated with lung metastasis, and predicts a poor prognosis. A, B. Representative images (200 × and 400 ×) of IHC staining and the quantitative comparison of IHC scores for ID1 expression in fibrous dysplasia, osteoblastoma, and osteosarcoma. ID1 expression was notably and significantly higher in osteosarcoma than in fibrous dysplasia and osteoblastoma. C. Higher IHC scores of ID1, which symbolized protein expression intensity, were found in patients with metastasis compared with those in patients without metastasis. D. A comparison of ID1 expression between low-grade osteosarcoma and high-grade osteosarcoma: The IHC scores of ID1 were significantly higher in Enneking stage III patients. **P < 0.01, ***P < 0.001, Scale bars: 50 μm.

Table 2. Correlations between ID1 expression and the clinicopathological features of patients with osteosarcoma

Characteristics	ID1 protein		Chi-square *P*-value[#]
	High expression	Low expression	
Age (years)			0.194
≤ 20	14	23	
21~30	8	5	
≥ 31	10	7	
Gender			0.582
Male	18	22	
Female	14	13	
Stage			0.025[*]
I	0	1	
II	21	31	
III	11	3	
Local recurrence			0.122
Yes	15	10	
No	17	25	
Distant metastasis			0.010[*]
Yes	16	7	
No	16	28	
Histological type			0.729
Osteoblastic	18	21	
Chondroblastic	9	11	
Fibroblastic	3	1	
Others	2	2	
Tumor size			0.065
< 8 cm	19	28	
≥ 8 cm	13	7	
Tumor location			0.410
Limbs	26	31	
Others	6	4	

[#]Pearson's χ^2 test; [*]with significant difference (P < 0.05).

used IHC staining of 67 human specimens to investigate the correlations between ID1 expression and the clinicopathological features of osteosarcoma. There were 40 males and 27 females, with a median age of 23.75 years old (range: 8-65). Twenty-five patients showed local recurrence, 14 patients had metastasis at diagnosis, and nine patients developed distant disease after treatment. The other clinicopathological features of the patients are listed in **Table 2**. We found that high expression of ID1 was significantly associated with advanced Enneking stage and metastasis (P < 0.05), but not with age, sex, pathological subtype, or location (**Table 2**). Accordingly, a high IHC score for ID1 was much more frequently observed in patients with an advanced Enneking stage and distant metastatic disease (**Figure 1C**, **1D**). In addition, higher ID1 expression was found in 40.43% (19/47) of patients with a smaller tumor size, in 40.48% (17/42) of patients without local recurrence, in 65% (13/20) of patients with a bigger tumor size, and in 60% (15/25) of patients with local recurrence; however, the differences were not statistically significant (P = 0.065 and 0.122) (**Table 2**). Furthermore, a Kaplan-Meier curve showed that high expression of ID1 was significantly related to poor overall survival in patients with osteosarcoma (P = 0.0395) (**Figure 2A**, **2B**). Taken together, these results showed that high expression of ID1 is positively associated with metastasis of osteosarcoma and is a potential biomarker of poor prognosis.

ID1 overexpression or knockdown does not have a significant effect on cell migration or invasion of osteosarcoma cells in vitro

The finding of high ID1 expression being positively correlated with metastasis in osteosarcoma prompted us to examine the effect of ID1 in regulating migration and invasion in osteosarcoma cells. First, we overexpressed ID1 using lentivirus transfection and knocked down ID1 expression using siRNAs in 143B and M/HOS osteosarcoma cell lines. Two effective target sequences of siRNAs were used and the

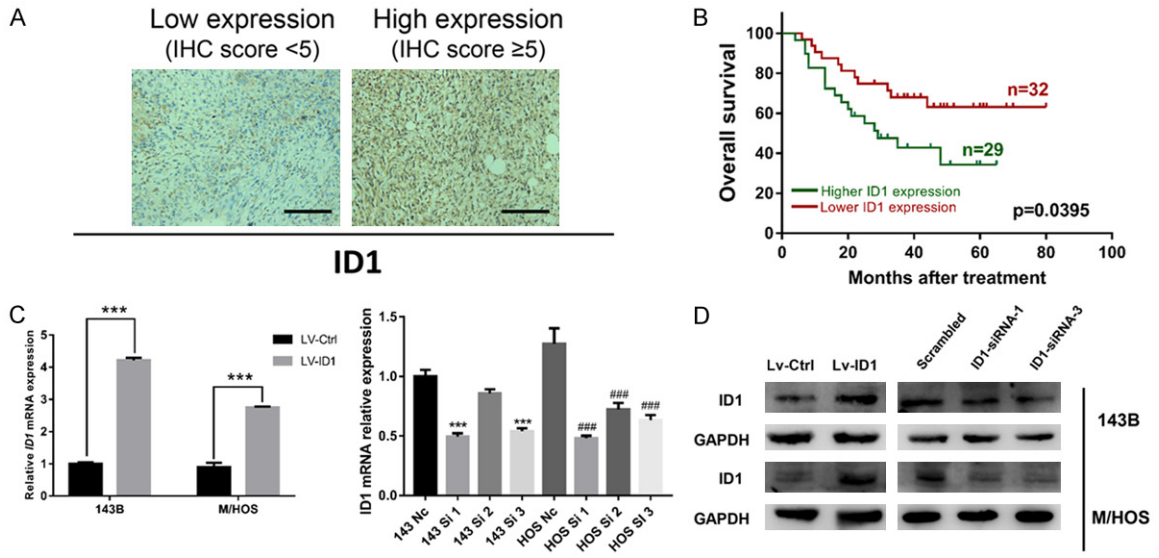
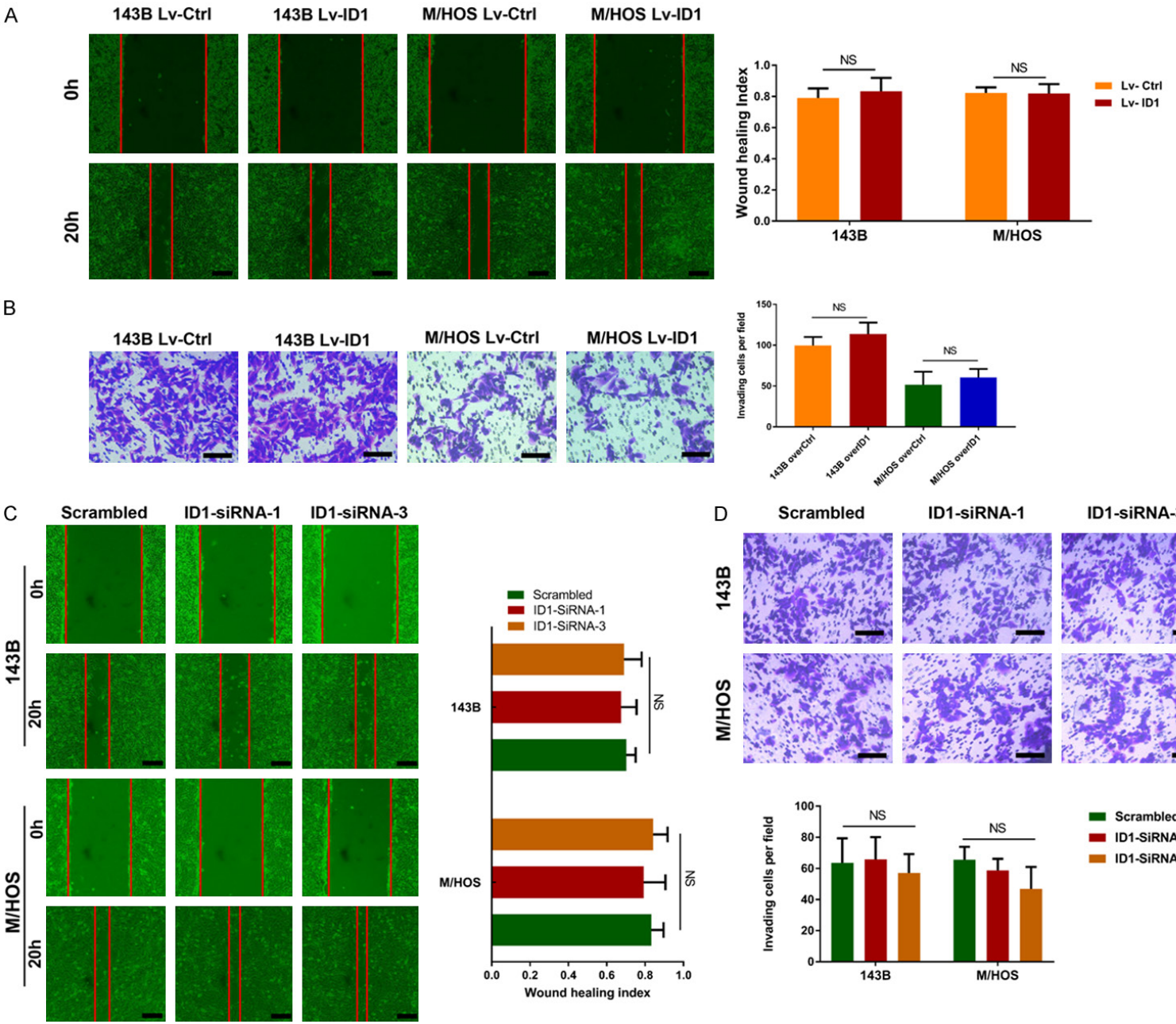


Figure 2. High expression of ID1 predicts a poor prognosis in patients with osteosarcoma and overexpressed or knocked down ID1 in osteosarcoma cells. A. Representative images (200 ×) of IHC for high or low expression staining of ID1 in human osteosarcoma tissues. IHC scores ≥ 5 were defined as high expression of ID1 (average score was 4.97). B. Kaplan-Meier curve showing that high expression of ID1 is significantly associated with poor prognosis and shorter overall survival (61 of 67 patients with exact follow-up data were included). Scale bars: 100 μm. ID1, inhibitor of differentiation or DNA binding 1; IHC, immunohistochemistry. C, D. Overexpressed ID1 expression using lentivirus transfection and knocked down ID1 expression using siRNAs in 143B and M/HOS osteosarcoma cell lines were confirmed by qPCR and Western blot analysis.

effect of ID1 overexpression and knockdown were both confirmed by qPCR and western blotting analysis (**Figure 2C**, **2D**). We then performed Transwell and wound healing assays to determine whether a change of ID1 level affected motility and the invasive ability of osteosarcoma cells. Surprisingly, overexpression or knockdown of ID1 had no significant effect on the migration or invasion of 143B and M/HOS cells (**Figure 3A-D**). MMPs are critical enzymes that promote the invasive ability of cancer cells [27] and there is evidence that ID1 could regulate the expression of MMPs in some type of cancers [28, 29]. In complementary experiments, we further used the R2 database (http://hgserver1.amc.nl) to investigate the correlation between ID1 and MMP-related genes in osteosarcoma samples. The results showed that there was only a weak positive correlation between ID1 and MMP2 ($r = 0.2561$, $P = 0.0037$) (**Figure 3E**). However, we could not find a consistent result in osteosarcoma cells in vitro: There was no significant change in the mRNA and protein expression of MMP2 with increased expression of ID1 in either of the osteosarcoma cell lines (**Figure 3F**), suggesting that the correlation between MMP2 and ID1 in tissues could potentially be attributed to the tumor microenvironment and not the direct functions of the cells. Meanwhile, the results supported the observation that ID1 had no obvious direct effect on the invasion of osteosarcoma cells in vitro. Collectively, these results suggested that although high expression of ID1 promoted lung metastasis in osteosarcoma, it may not be attributed to increased cell migration and invasion.

Overexpression of ID1 inhibits anoikis of osteosarcoma cells through the mitochondrial apoptosis pathway suppression in vitro

We next hypothesized that the possible mechanism by which ID1 regulates osteosarcoma cell metastasis might involve anoikis, which is known as an important process in tumor distant metastasis [30]. A previous study showed that ID1 could improve apoptosis resistance in osteosarcoma cells under adherence conditions [21]. Accordingly, we aimed to identify whether ID1 could influence anoikis of osteosarcoma cells in vitro. When cultured in ultra–low attachment 6-well plates as suspension cultures for 48 hours, 143B and M/HOS cells overexpressing ID1 showed significantly atten-


A
143B Lv-Ctrl
143B Lv-ID1
M/HOS Lv-Ctrl
M/HOS Lv-ID1
0h
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Wound healing Index
NS
Lv- Ctrl
Lv- ID1
143B
M/HOS
B
Invading cells per field
143B overCtrl
143B overID1
M/HOS overCtrl
M/HOS overID1
C
Scrambled
ID1-siRNA-1
ID1-siRNA-3
Wound healing index
D

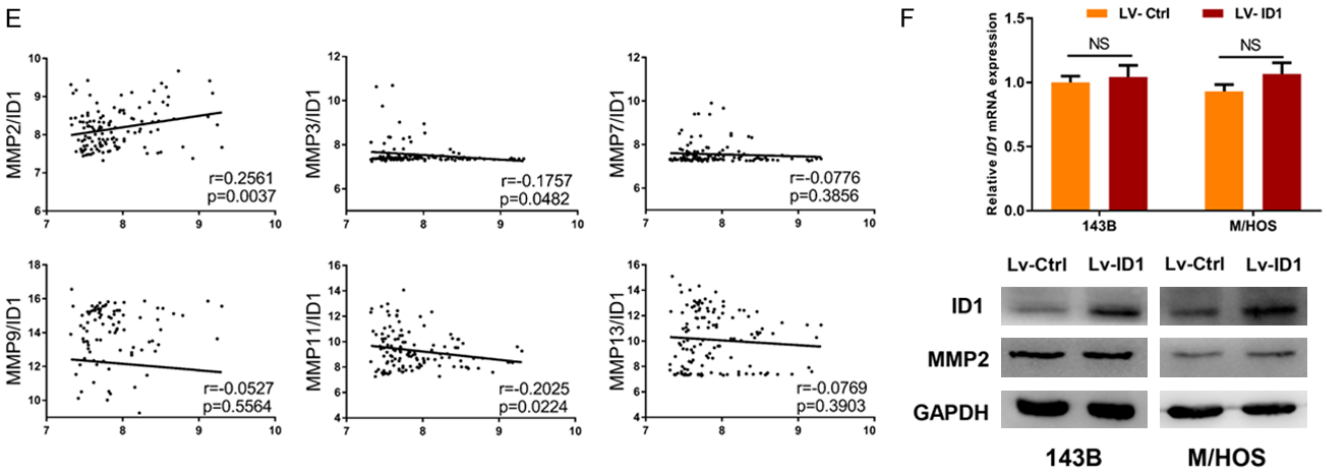
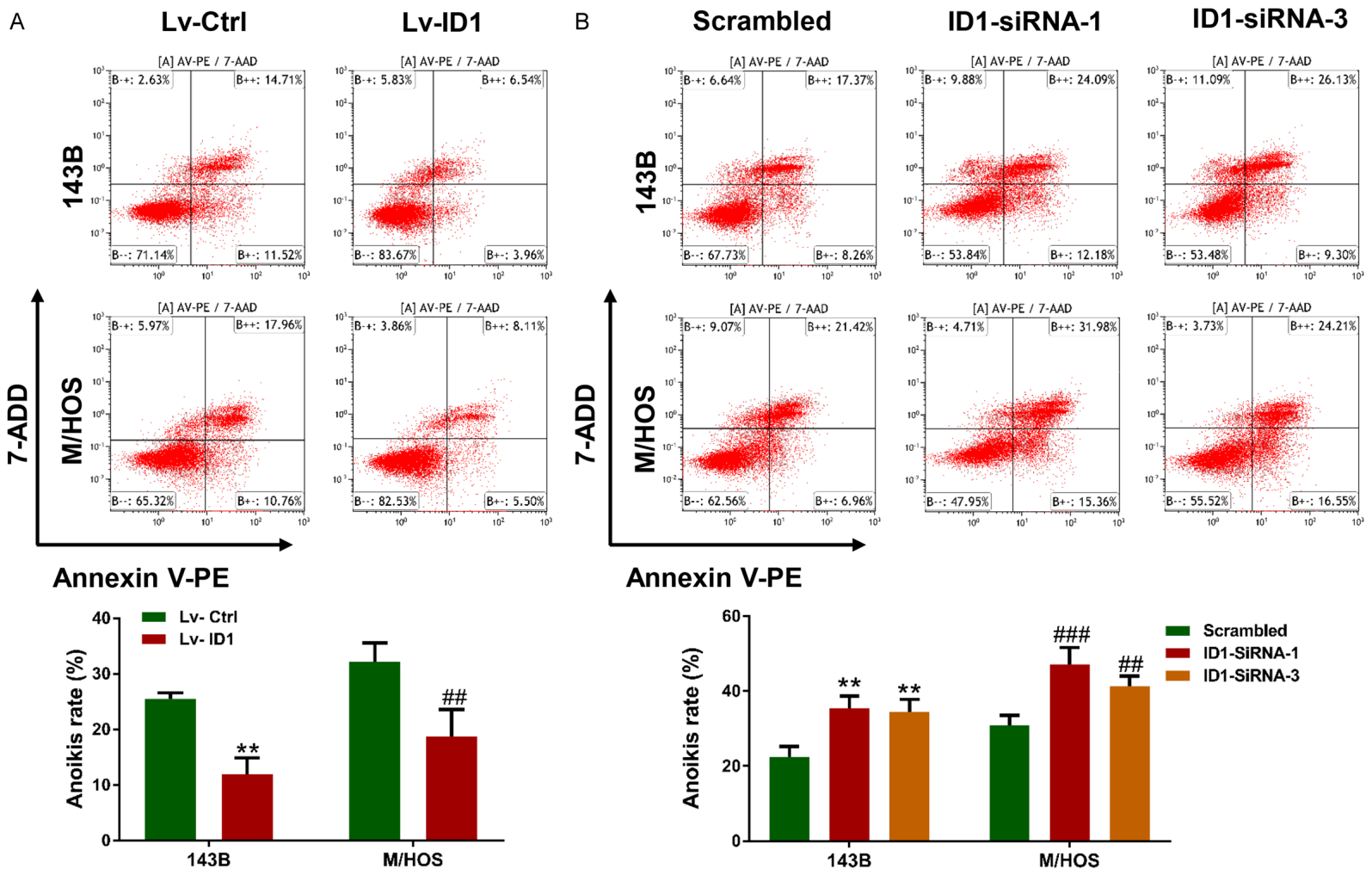


Figure 3. ID1 has no significant effect on osteosarcoma cell migration and invasion. A. Wound healing assays were conducted for cells transfected with control (Lv-Ctrl) and ID1-overexpression (Lv-ID1) lentiviruses in 143B or M/HOS cells for 20 h. B. Transwell invasion assay indicating that overexpression of ID1 does not affect 143B or M/HOS osteosarcoma cells invasion in vitro. C, D. ID1 knockdown by siRNAs also does not affect osteosarcoma cell migration and invasion significantly. E. Pearson correlation analysis between ID1 and common MMP genes mRNA expression in human osteosarcoma tissues by analyzing the R2 gene expression database (http://hgserver1.amc.nl). F. Representative images of western blotting analysis of ID1 and MMP2 in 143B and M/HOS cells. Cells were treated with or without ID1-overexpression. NS, no significant difference; Scale bars: 200 μm.


A
Lv-Ctrl
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143B
M/HOS
7-ADD
Annexin V-PE
[A] AV-PE / 7-AAD
B-+: 2.63%
B++: 14.71%
B--: 71.14%
B+-: 11.52%
B-+: 5.83%
B++: 6.54%
B--: 83.67%
B+-: 3.96%
B-+: 5.97%
B++: 17.96%
B--: 65.32%
B+-: 10.76%
B-+: 3.86%
B++: 8.11%
B--: 82.53%
B+-: 5.50%
Lv- Ctrl
Lv- ID1
Anoikis rate (%)
40
30
20
10
0
**
##
143B
M/HOS
B
Scrambled
ID1-siRNA-1
ID1-siRNA-3
B-+: 6.64%
B++: 17.37%
B--: 67.73%
B+-: 8.26%
B-+: 9.88%
B++: 24.09%
B--: 53.84%
B+-: 12.18%
B-+: 11.09%
B++: 26.13%
B--: 53.48%
B+-: 9.30%
B-+: 9.07%
B++: 21.42%
B--: 62.56%
B+-: 6.96%
B-+: 4.71%
B++: 31.98%
B--: 47.95%
B+-: 15.36%
B-+: 3.73%
B++: 24.21%
B--: 55.52%
B+-: 16.55%
Scrambled
ID1-SiRNA-1
ID1-SiRNA-3
60
40
20
0
###
##
**
**

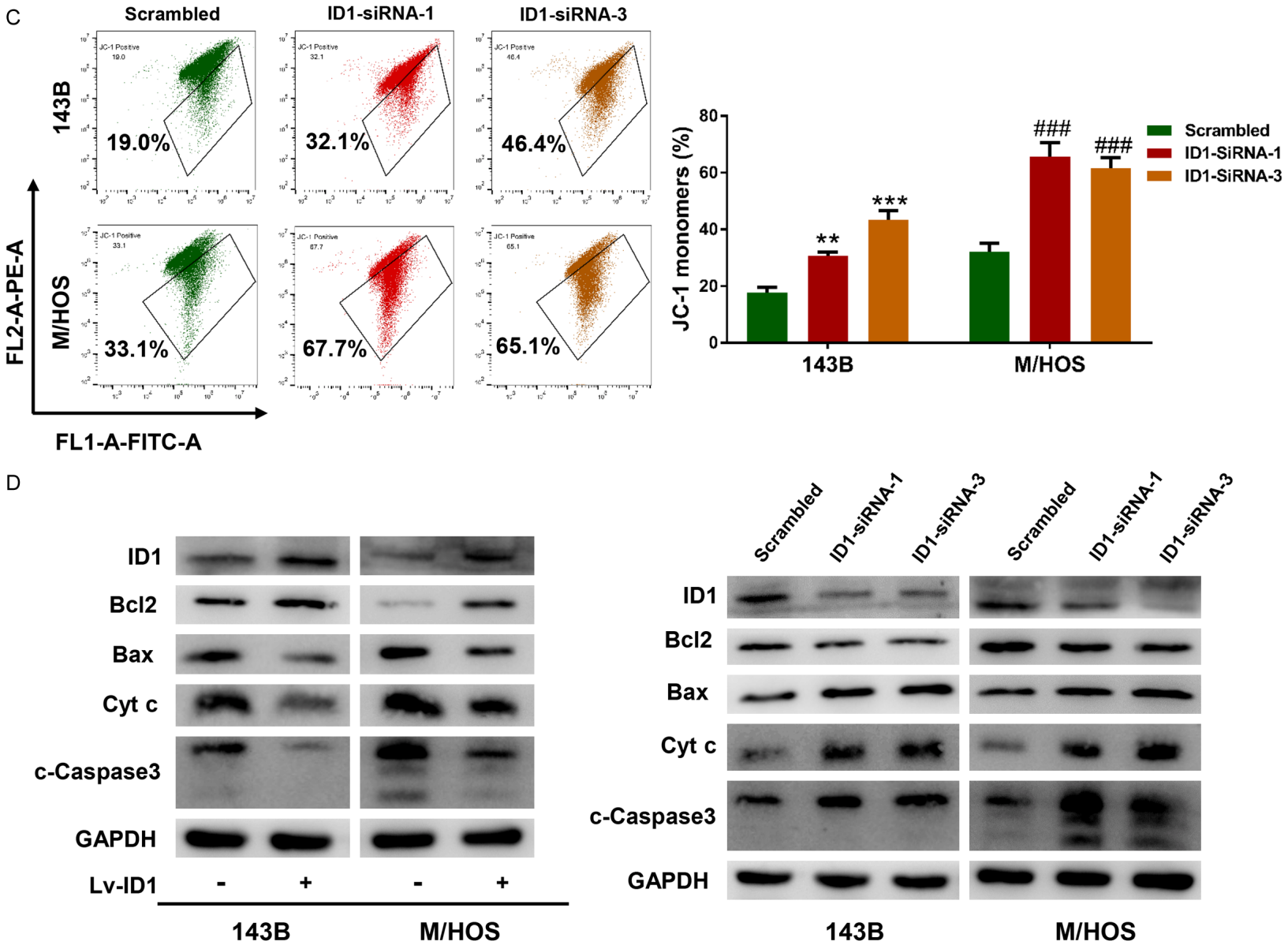


Figure 4. ID1 regulates sensitivity of anoikis in osteosarcoma cells through mitochondrial apoptosis pathway suppression in vitro. A. Annexin V-PE/7-ADD assay indicating that after 48 hours of culture, the anoikis rate of cells with ID1 overexpression is much lower than that of the control cells. B. Knockdown of ID1 expression by

siRNAs increased the anoikis rate of 143B and M/HOS cells significantly. C. Representative images and quantification of mitochondrial membrane potential (JC-1). Cells with or without ID1 silencing were cultured for 48 hours and then stained with JC-1 solution for flow cytometry analysis. D. Representative images of the western blotting analysis of ID1, BCL2, BAX, cleaved-caspase 3, and Cytochrome versions in 143B and M/HOS cells. Cells were subjected to ID1 overexpression or silencing. **P < 0.01, ***P < 0.001, compared with the 143B Ctrl group, ##P < 0.01, ###P < 0.01, compared with the M/HOS Ctrl group.

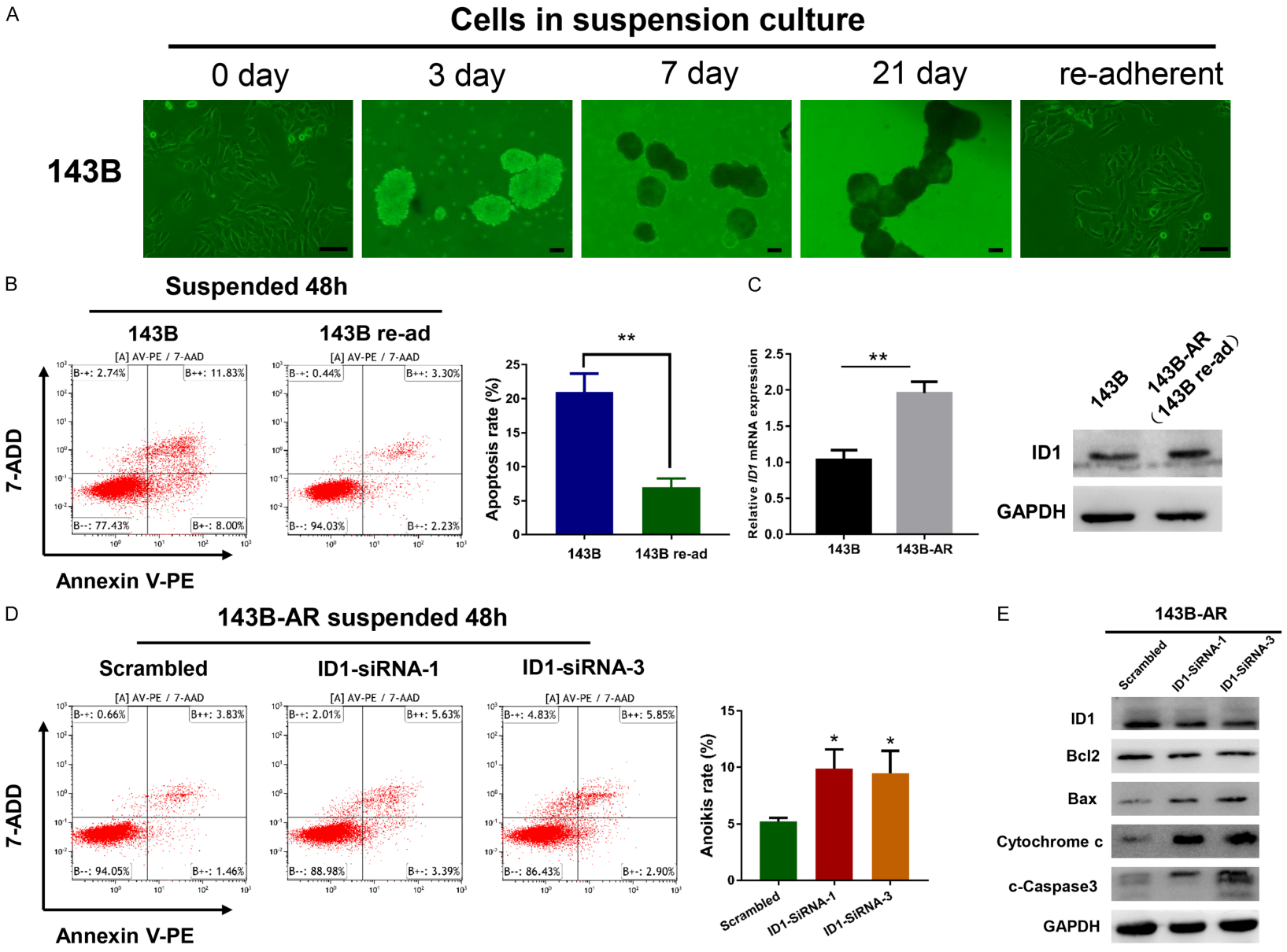
uated anoikis compared with that in their respective control groups (**Figure 4A**). By contrast, treatment with ID1-siRNAs increased the percentage of anoikis cells (**Figure 4B**). These results suggested that ID1 could increase anoikis insensitivity in osteosarcoma cells, which might provide several advantages for the metastatic behavior of osteosarcoma. Moreover, ID1 has been implicated in the intrinsic apoptosis pathway (mitochondrial), which has been described as a key regulator of anoikis [31]. To identify the apoptotic signaling pathway that underlies ID1-mediated anoikis, we first applied JC-1 to measure the mitochondrial membrane potential by flow cytometry. The results showed a notable increase in the loss of mitochondrial membrane potential, reflected as increased numbers of JC-1 monomers, in cells treated with the ID1 siRNAs compared with that in the control cells (**Figure 4C**). Then, we detected the dynamic changes of mitochondrial apoptotic proteins. The results demonstrated that overexpression of ID1 not only increased the level of the anti-apoptotic protein BCL2, but also reduced the level of the pro-apoptotic proteins BAX, cytochrome c, and cleaved caspase-3 in the 143B and M/HOS cells under suspension culture, which was reversed by knockdown of ID1 (**Figure 4D**). Thus, these results indicated that ID1 could regulate the anoikis of osteosarcoma cells via the mitochondrial apoptosis pathway.

Crucial involvement of ID1 in osteosarcoma cells acquiring anoikis-resistance

Our results showed that ID1 potentiated the anti-anoikis property of osteosarcoma cells; therefore, we sought to determine whether ID1 is involved in the process by which osteosarcoma cells acquire anoikis-resistance. We established anoikis-resistant cell models by sequential cycles of culture switching under adherent and suspended conditions [8]. We found that the 143B and M/HOS cells gathered into clusters by degrees in the suspension culture and became denser over time. The appearance of the re-adherent cells was not visibly different compared with that of original untreated cells under an inverted phase contrast light microscope (**Figure 5A** and **5F**). However, after attachment was inhibited for 48 hours, the re-adherent 143B cells became resistant to apoptosis. A low percentage of apoptotic cells (6.97 ± 0.74%) was found in re-adherent 143B cells, while a much higher percentage apoptotic cells (20.99 ± 1.57%) was found for 143B cells under suspension culture for 48 hours (**Figure 5B**). The significantly decreased anoikis rate ($P < 0.05$) indicated that we had successfully established anoikis-resistant 143B cells (143B-AR). Moreover, we observed increased ID1 mRNA and protein expression in 143B-AR cells compared with that in 143B cells (**Figure 5C**). Annexin V-PE/7-ADD analysis showed that the anoikis resistance of 143B-AR cells was partly reversed using siRNA-mediated knockdown of ID1 (**Figure 5D**). In addition, consistent with the flow analysis results, western blotting analysis revealed decreased expression of BCL2 and an accumulation of BAX, cytochrome c, and cleaved-caspase 3 in the ID1-silenced cells (**Figure 5E**), which indicated that the activated mitochondrial apoptotic pathway could be attributed to the decreased expression of ID1 in 143B-AR cells. Interestingly, we failed to established an anoikis-resistant model in M/HOS cells (**Figure 5G**) and were also unable to detect a notable change in the expression of ID1 between re-adherent M/HOS cells and original M/HOS cells (**Figure 5H**). Taken together, these findings suggested that increased ID1 expression might be a hallmark of osteosarcoma cells that have acquired anoikis-resistance during circulation.

ID1 promotes osteosarcoma cells lung metastasis and ID1 silencing partly reverses the increased metastatic ability of anoikis-resistance cells in vivo

To further investigate whether ID1-induced anoikis inhibition contributes to metastasis in vivo, we constructed a mouse lung metastasis model by injecting M/HOS cells stably overexpressing ID1 and control M/HOS cells into the tail vein of the mice. In line with our in vitro findings, overexpression of ID1 notably increased


A
Cells in suspension culture
0 day
3 day
7 day
21 day
re-adherent
143B
B
Suspended 48h
143B
143B re-ad
[A] AV-PE / 7-AAD
B-+: 2.74%
B++: 11.83%
B--: 77.43%
B+-: 8.00%
B-+: 0.44%
B++: 3.30%
B--: 94.03%
B+-: 2.23%
7-ADD
Annexin V-PE
Apoptosis rate (%)
143B
143B re-ad
C
Relative ID1 mRNA expression
143B
143B-AR
143B-AR (143B re-ad)
ID1
GAPDH
D
143B-AR suspended 48h
Scrambled
ID1-siRNA-1
ID1-siRNA-3
B-+: 0.66%
B++: 3.83%
B--: 94.05%
B+-: 1.46%
B-+: 2.01%
B++: 5.63%
B--: 88.98%
B+-: 3.39%
B-+: 4.83%
B++: 5.85%
B--: 86.43%
B+-: 2.90%
Anoikis rate (%)
E
143B-AR
Scrambled
ID1-SiRNA-1
ID1-SiRNA-3
ID1
Bcl2
Bax
Cytochrome c
c-Caspase3
GAPDH

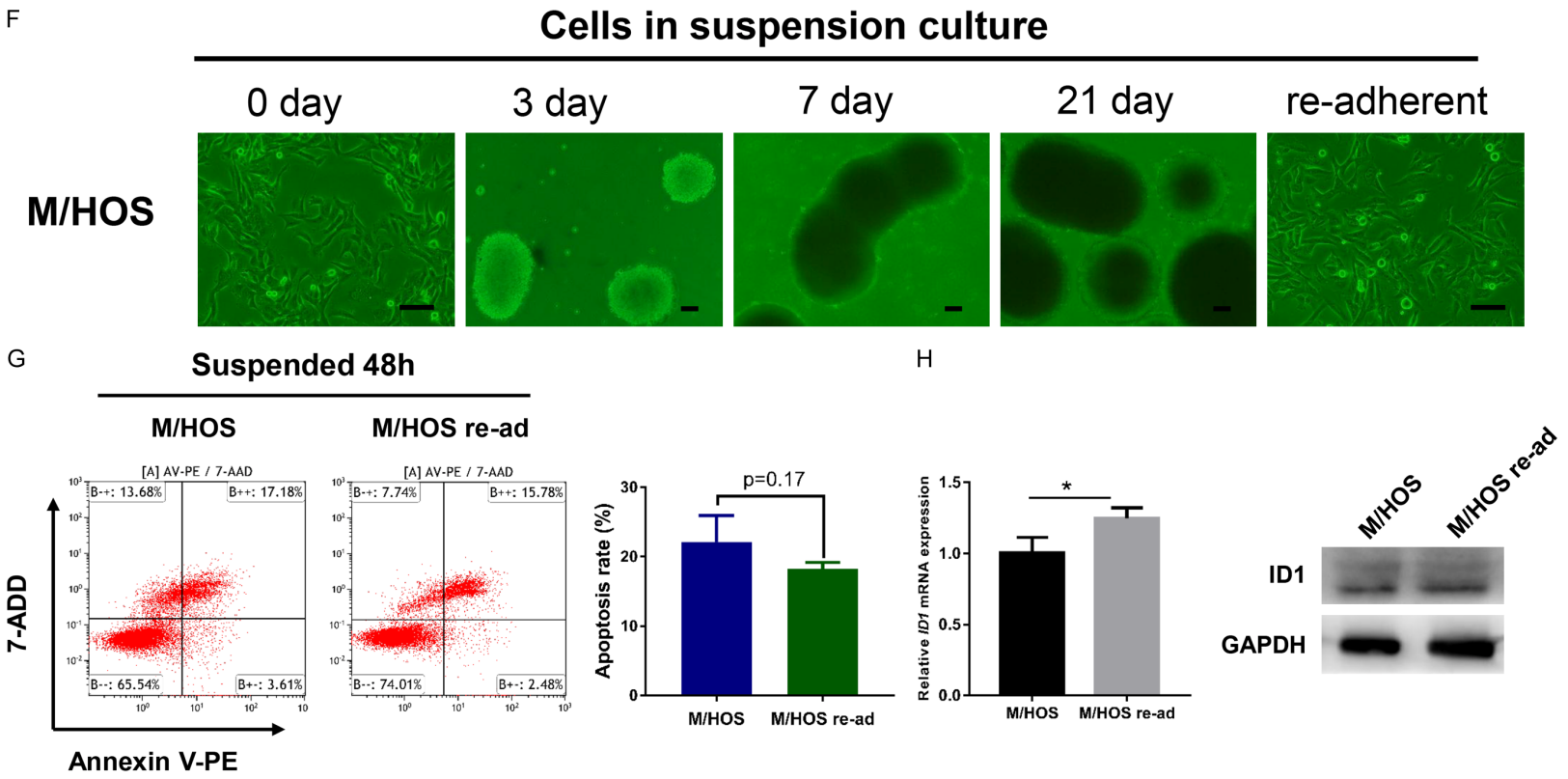


Figure 5. Crucial involvement of ID1 in osteosarcoma cells to acquire anoikis-resistance. A and F. Morphology of 143B and M/HOS clusters cultured in ultra low attachment 6-well plates at the indicated times captured under an inverted light microscope, scale bars: 100 μm. B. Annexin V-PE/7-ADD assay indicating that after culture for 48 hours, the apoptotic rate of 143B re-ad cells (143B-AR) was much lower than that of 143B cells, $^{**}P < 0.01$. C. ID1 mRNA and protein expression was significantly higher in 143B-AR cells compared with that in 143B cells, $^{**}P < 0.01$. D. Anoikis resistance in 143B-AR cells was partly reversed by siRNA-mediated knockdown of ID1, $^{*}P < 0.05$, compared with the scrambled group. E. Representative images of western blotting analysis of ID1, BCL2, BAX, Cytochrome c, and cleaved-Caspase3 in 143B-AR cells treated as indicated. G. Annexin V-PE/7-ADD assay indicates there is no significant change of the apoptotic rate after suspended for 48 hours between M/HOS re-ad cells and M/HOS cells. H. ID1 mRNA and protein expression was found no significant change in M/HOS re-ad cells compared to M/HOS cells (Although there is a somehow significant increased mRNA expression of ID1 in M/HOS re-ad cells ($^{*}P < 0.05$), but it is always thought to be no practical significance when the relative fold change of the mRNA expression is less than 1.5 times).

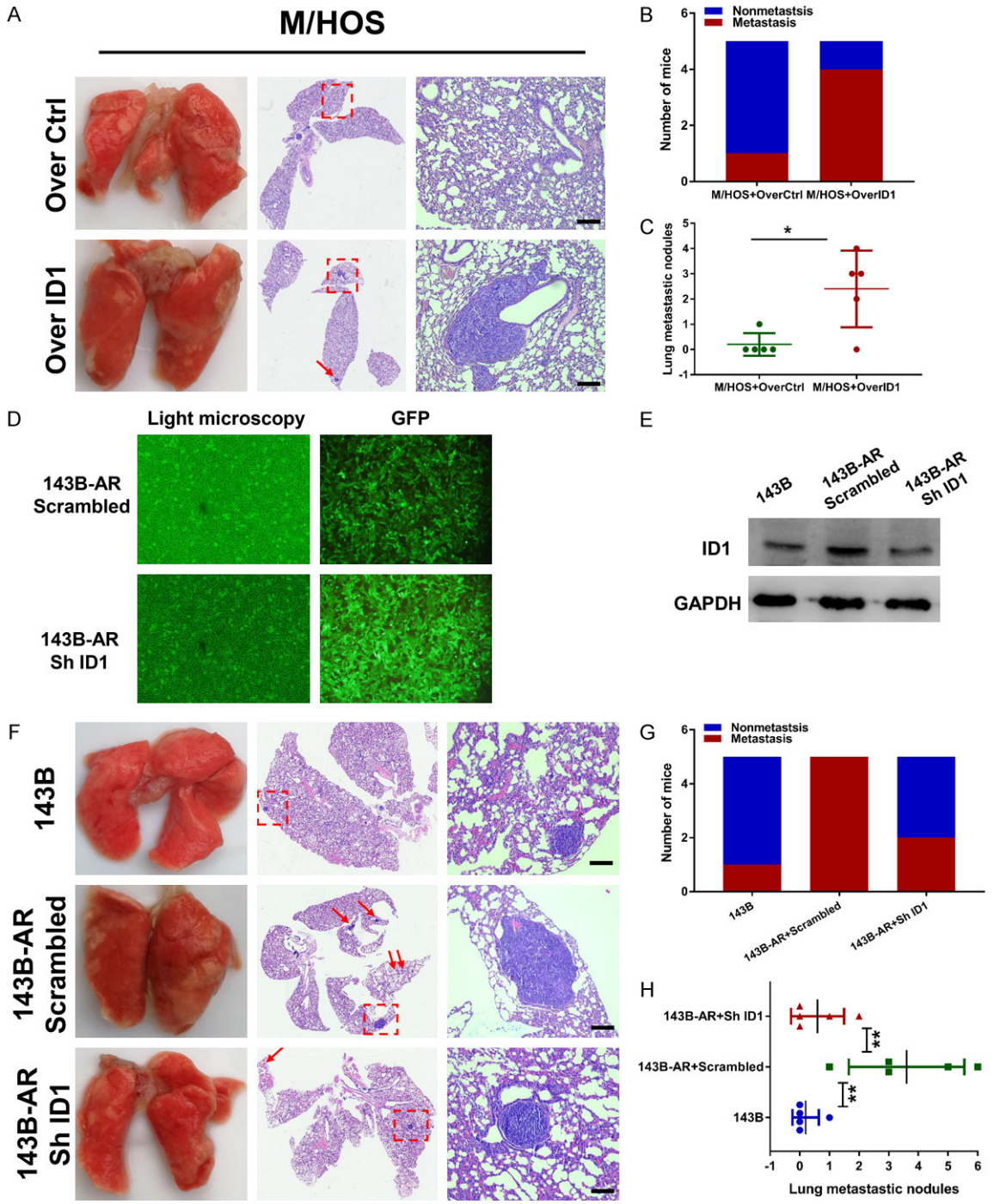


Figure 6. ID1 promotes osteosarcoma cells lung metastasis and ID1 silencing partly reverses the increased metastatic ability of anoikis-resistance cells in vivo. M/HOS and 143B cells were transfected as indicated and injected into the tail vein of nude mice to establish an in vivo model of lung metastasis. A and F. Representative macroscopic and microscopic images (H&E staining) of mouse lungs as indicated. Red dotted line sections were enlarged and red arrows indicate confirmed metastatic lesions. B and G. Percentage of mice bearing lung metastases in each group (n = 5). C and H. Numbers of lung metastatic nodules quantified on H&E-stained lung sections. D and E. ID1 was successfully silenced in 143B-AR cells. *P < 0.05, **P < 0.01, scale bars: 100 μm.

was more frequently observed in high grade and aggressive subgroups in several other malignancies, such as nasopharyngeal carcinoma, myeloid leukemia, and cervical cancer

the ability of M/HOS cells to produce lung metastases, as reflected by the increased metastasis incidence and greater number of tumor metastatic nodules in the lungs of the mice injected the cells overexpressing ID1 (**Figure 6A-C**). Moreover, anoikis-resistant cells had a much greater capacity to establish lung metastases compared with their original forms. However, using the lentiviral siRNA vector to stably silence ID1 in 143B-AR cells (**Figure 6D**, **6E**) markedly decreased the metastatic rate and the number of metastatic nodules were markedly decreased (143B-AR ShID1 group; **Figure 6F-H**), which indicated that ID1 silencing at least partly reversed the increased metastatic ability of anoikis-resistant cells in vivo. Together with our in vitro findings, we demonstrated that ID1 has a significant effect on osteosarcoma metastasis by inhibiting anoikis.

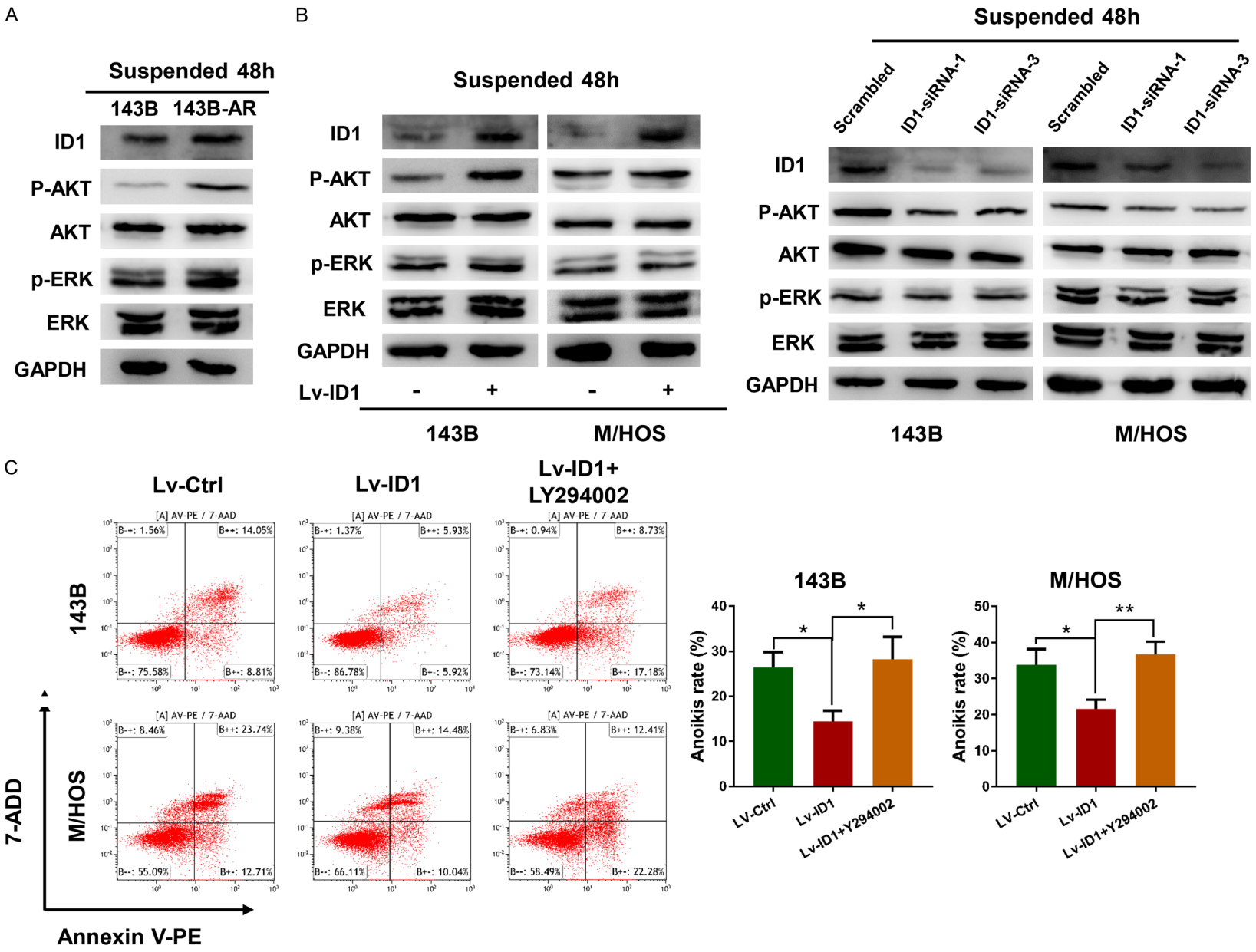
ID1 induces anoikis-resistance in osteosarcoma cells via activating the PI3K/AKT pathway

Previous studies suggested that ID1 expression could regulate the PI3K/AKT and MEK/ERK signaling pathways [21, 32-34]. In view of the important roles of the PI3K/AKT and ERK pathways in anoikis resistance and mitochondrial apoptotic signaling regulation [30], we further investigated how ID1 mediated anoikis in osteosarcoma cells. First, we measured the levels of AKT, ERK, and their phosphorylated forms in 143B and 143B-AR cells. The results revealed that the levels of the phosphorylated forms of AKT and ERK under conditions of ID1 overexpression were significantly increased in 143B-AR cells after suspension culture for 48 hours compared with those in 143B cells (**Figure 7A**), suggesting that ID1, the PI3K/AKT pathway, and the ERK pathway participate in inducing osteosarcoma cells together to become anoikis resistant. However, after the indicated treatment of 143B and M/HOS cells under suspension conditions for 48 hours, the results showed that overexpression of ID1 did not affect the total AKT, ERK and p-ERK levels, but significantly increased the level of p-AKT (**Figure 7B**). In parallel, knockdown of ID1 led to significantly decreased levels of p-AKT, which suggested that ID1 preferentially acts via PI3K/AKT activation rather then ERK signaling to induce anoikis insensitivity. Then, we applied LY294002, a specific inhibitor of the PI3K/AKT pathway, in ID1-overexpressing cells, to further assess whether the PI3K/AKT pathways underlies ID1-mediated anoikis. Strikingly, following LY294002 treatment, a marked increase in cells undergoing anoikis was detected in ID1-overexpressing cells using flow cytometry (**Figure 7C**). This result implied that activation of the PI3K/AKT signaling pathway was essential for ID1-induced anoikis inhibition. In addition, accompanied by a significant increase in anoikis caused by PI3K/AKT inhibition, we could also observe consistently higher expression of pro-apoptotic proteins and lower expression of anti-apoptotic proteins of the intrinsic apoptotic signaling pathway (**Figure 7D**), suggesting that the mitochondrial pathway acts as a functional downstream effector of the ID1/PI3K/AKT axis. Thus, these results highlighted the relevance of ID1 and the PI3K/AKT signaling pathway as a regulatory mechanism of anoikis of osteosarcoma cells.

Discussion

Osteosarcoma is one of the most common causes of cancer-related death in young adolescents [22]. The high mortality of osteosarcoma can be attributed to metastases; however, there are few effective treatments for distant metastasis [35]. Thus, there is an urgent need for new insights into the molecular mechanism of metastasis in osteosarcoma. ID1, an HLH family member, has potential effects on development and the cell cycle, and is reported to regulate tumorigenicity in several malignant tumors, including osteosarcoma [36]. Previous studies provided preliminary evidence that ID1 may act as an oncogene in osteosarcoma [20, 21]. Although certain tumor-promoting effects are associated with ID1 expression in osteosarcoma, such as anti-apoptosis and promotion of proliferation [21], the relationship between ID1 and metastasis remains poorly understood.

ID1 mRNA is overexpressed in human osteosarcoma tissues [20]; however, overexpression of the protein has not been reported. In the present study, IHC indicated that the ID1 protein was highly abundant in osteosarcoma samples compared with that in benign bone tumor samples. Further analyses demonstrated that high expression of ID1 was positively correlated with an advanced Enneking stage and distant metastasis. Similarly, higher expression of ID1


A
Suspended 48h
143B
143B-AR
ID1
P-AKT
AKT
p-ERK
ERK
GAPDH
B
Suspended 48h
ID1
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AKT
p-ERK
ERK
GAPDH
Lv-ID1
-
+
-
+
143B
M/HOS
Suspended 48h
Scrambled
ID1-siRNA-1
ID1-siRNA-3
Scrambled
ID1-siRNA-1
ID1-siRNA-3
ID1
P-AKT
AKT
p-ERK
ERK
GAPDH
143B
M/HOS
C
Lv-Ctrl
Lv-ID1
Lv-ID1+
LY294002
143B
M/HOS
7-ADD
Annexin V-PE
[A] AV-PE / 7-AAD
B-+: 1.56%
B++: 14.05%
B--: 75.58%
B+-: 8.81%
B-+: 1.37%
B++: 5.93%
B--: 86.78%
B+-: 5.92%
B-+: 0.94%
B++: 8.73%
B--: 73.14%
B+-: 17.18%
B-+: 8.46%
B++: 23.74%
B--: 55.09%
B+-: 12.71%
B-+: 9.38%
B++: 14.48%
B--: 66.11%
B+-: 10.04%
B-+: 6.83%
B++: 12.41%
B--: 58.49%
B+-: 22.28%
143B
M/HOS
Anoikis rate (%)
LV-Ctrl
Lv-ID1
Lv-ID1+Y294002

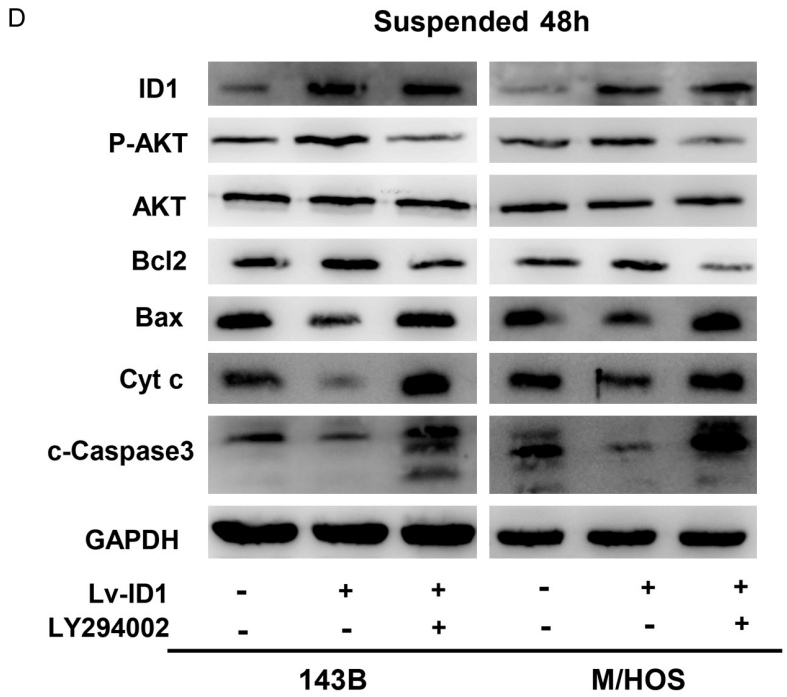

Figure 7. ID1 induces anoikis-resistance in osteosarcoma cells via activating the PI3K/AKT pathway to suppress mitochondrial apoptotic signals. A. Representative images of the western blotting analysis of ID1, ERK, p-ERK, AKT, and p-AKT levels in 143B and 143B-AR cells after 48 hours suspension. B. Representative images of the western blotting (WB) analysis of ID1, AKT, ERK, and their phosphorylated versions in 143B and M/HOS cells. Cells were treated with ID1-overexpression or knockdown. C. The ID1-induced-insensitive to anoikis were altered by PI3K/AKT inhibitors (LY294002). *P < 0.05, **P < 0.01. D. Representative images of the WB analysis of ID1, BCL2, BAX, cleaved-caspase 3, Cytochrome c, AKT and the phosphorylated versions in 143B and M/HOS cells.

[37-39]. Our results also demonstrated that ID1 could be a useful biomarker for prognosis in patients with osteosarcoma. Therefore, examining ID1 protein expression by IHC could represent an easy and effective way to identify patients at increased risk of distant metastasis, progression, and poor prognosis. These findings suggested a novel role for ID1 in the underlying biological mechanism of metastasis in human osteosarcoma.

ID1 is not only importantly in cancer cell proliferation, but also is involved in transformation of cancer cells to an invasive phenotype, such as in migration, invasion, and the epithelial-mesenchymal transition (EMT) [18, 26, 32]. Although a previous study focused on the role of the ID1 in tumor growth in osteosarcoma [21], no study has investigated the potential relationship between ID1 and the metastatic behavior of osteosarcoma cells. Considering our data from human specimens, we expected that ID1 would facilitate the invasive phenotype of osteosarcoma cells similar to previous studies in other malignant tumor cells [17, 29]. Surprisingly, ID1 overexpression or knockdown had no significant effect on the cell migration and invasion in two kinds of osteosarcoma cells. Although this could be partially explained as a consequence of heterogeneity of different kinds of tumors, these results did not correlate with those from human osteosarcoma tissues. Osteosarcoma cell metastasis results from a complex series procedures including not only cell mobility and invasion, but also resistance to anoikis.

Therefore, we decided to investigate anoikis resistance and its potential association with ID1. Osteosarcoma cells are sensitive to anoikis and after sequential cycles of adherence and suspended culture, some osteosarcoma cells can alter their sensitivity to anoikis to become resistant. Emerging evidence suggests that anoikis resistance is a crucial process for osteosarcoma cells to acquire a metastatic phenotype [11-13, 15]. In the present study, we showed that overexpression of ID1 significantly decreased the sensitivity of osteosarcoma cells to anoikis. Indeed, the effect of ID1 in facilitating lung metastasis was further confirmed by our in vivo experiments, in which osteosarcoma cells were injected into mouse tail veins to simulate the anoikis situation. Moreover, we found that ID1 acted as a control gene in the process of anoikis resistance in 143B cells, but not in M/HOS cells. We speculated that the ability to acquire anoikis resistance varied among different cell lines under our culture conditions. More importantly, our results showed that anoikis-resistant osteosarcoma cells displayed advanced metastatic ability and that knockdown of ID1 expression could at least partly restore the anoikis sensitivity of anoikis-resistant cells to inhibit them from forming distant metastasis in vivo. These findings showed that ID1 is a potential molecular target to restrict osteosarcoma cells with high metastatic potential in localized tumors. However, anoikis-resistant subpopulations of osteosarcoma cells also display significant chemoresistance and angiogenesis [12, 15]. Thus, further studies are needed to confirm the influence of ID1 on the characteristics of anoikis-resistant cells.

Accumulating evidence suggests that the SRC, PI3K/AKT, MAPK, FAK, and integrin pathways are involved in conferring anoikis resistance on osteosarcoma cells [9, 14, 30, 40, 41]. Meanwhile, ID1 activity has been reported to correlate with the SRC, PI3K/AKT, and MAPK/ERK pathways [21, 32, 42], prompting us to confirm these potential mechanisms during anoikis. However, we decided not to further investigate the correlation with SRC because a previous study showed that inhibiting SRC phosphorylation did not alter metastatic potential of osteosarcoma cells in vivo [43]. Thus, we first demonstrated that the AKT and ERK pathways were both involved in the anoikis and were notably activated in anoikis-resistant cells. Moreover, we found that PI3K/AKT activation, but not ERK activation, was an essential process for ID1-mediated induction of anoikis insensitivity during suspended culture, suggesting that ERK may not be a direct downstream target of ID1. Furthermore, numerous reports have highlighted the importance of the interplay of triggering two apoptotic pathways, the intrinsic (mitochondrial) pathway and extrinsic pathway, during anoikis [30]. In addition, ID1 can regulate the expression of BCL2 family proteins, which play key roles in the mitochondrial pathway [44, 45]. In the present study, we demonstrated that ID1 regulates anoikis by upregulating the anti-apoptotic signal BCL2, and decreasing BAX expression, subsequently

maintaining the mitochondrial membrane potential, inhibiting cytochrome c release from the apoptosome, and finally impeding the activation of caspase-3 to suppress anoikis. These changes to the mitochondrial pathway are dependent on the PI3K/AKT pathway.

In conlusion, the results of present study demonstrated that ID1 promotes anoikis resistance of osteosarcoma cells to facilitate distant metastasis via inhibiting the PI3K/AKT-dependent mitochondrial pathway. Furthermore, ID1 protein expression is biomarker of poor prognosis in osteosarcoma. Importantly, knockdown of ID1 expression could restore the anoikis sensitivity of osteosarcoma cells to stop them from metastasizing. Therefore, our results highlight a novel role for ID1 as a potential target for metastatic potential osteosarcoma and provide insights into the underlying mechanism of anoikis resistance and metastasis of osteosarcoma.

Acknowledgements

The authors thank Professor Xue-Feng Tang for her helpful advice for this study. We would also like to thank the native English-speaking scientists of Elixigen Company (Huntington Beach, California) for editing our manuscript. Moreover, we would like to thank the Institute of Central Laboratory of Xinqiao Hospital for providing some of the experimental apparatus. We also thank Ms Xing Rong, Mr. Jia-Sheng Ye, and Ms Ya-Li Wang (Xinqiao Hospital) for their assistance with the immunohistochemistry techniques. This work was supported by the National Natural Science Foundation of China (NSFC) [grant number 81672653] and the Chongqing Science and Technology Committee Foundation, China [grant number cstc2015jcyj BX0067].

Disclosure of conflict of interest

None.

Address correspondence to: Dian-Ming Jiang, Department of Orthopedic Surgery, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, People's Republic of China. E-mail: jdm571026@vip.163.com; Qiao-Nan Guo, Department of Pathology, Xinqiao Hospital, Army Medical University, Chongqing 400037, People's Republic of China. Tel: +086 023 68755641; E-mail: qiaonan-85@263.net

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