RNA interference-mediated knockdown of Livin suppresses cell proliferation and invasion and enhances the chemosensitivity to cisplatin in human osteosarcoma cells

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Abstract. Livin is a novel member of the inhibitor of apoptosis protein (IAP) family that has been reported to be overexpressed in a variety of human malignancies, including osteosarcoma. However, the potential roles of Livin in tumorigenesis have not been elucidated. In the present study, we employed RNA interference (RNAi) technology to suppress endogenous Livin expression in osteosarcoma cells and successfully generated a U2-OS cell line with stably knockdown of Livin. Functional analysis showed that knockdown of Livin significantly reduced cell proliferation, colony formation, and invasion and migration capacities of U2-OS cells *in vitro*. Moreover, specific downregulation of Livin led to cell cycle arrest at the G0/G1 phase and eventual apoptosis. Meanwhile, western blot analysis revealed that cells with stably knockdown of Livin showed decreased expression levels of Cyclin D1, Bcl-2, matrix metalloproteinase (MMP)-2 and MMP-9, but increased expression levels of activated Caspase-3, Bax and cleaved poly (ADP-ribose) polymerase (PARP) compared to those transfected with a control vector. We also observed that suppression of Livin expression in osteosarcoma cells increased their chemosensitivity to cisplatin. Taken together, our data suggest that Livin is involved in tumorigenesis of human osteosarcoma and may serve as a promising therapeutic target for osteosarcoma.

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Introduction

Osteosarcoma is the most frequently diagnosed primary malignant bone tumor in children and adolescents, accounting for an estimated 2.4% of all malignancies in pediatric patients (1). The overall 5-year survival rate for osteosarcoma patients has improved from less than 20% in the early 1960s to around 70% by the mid-1980s, largely as a consequence of advances in medical treatments (i.e., chemotherapy and limb salvage surgery) (2). However, there have been no continuous improvements over the past twenty years, and the 5-year survival rate for patients with the metastatic disease remains approximately 20% (3,4). Therefore, the identification of key regulators that govern progression and malignant behavior of osteosarcoma may provide insights into the development of novel therapeutic strategies for this malignancy.

Livin [also known as melanoma inhibitor of apoptosis protein (ML-IAP) or kidney inhibitor of apoptosis protein (KIAP)] belongs to the inhibitor of apoptosis protein (IAP) family that also includes C-IAP1, C-IAP2, NAIP, Survivin, X-linked IAP (XIAP), Bruce and IAP-like protein (ILP)-2 (5,6). Similar to other IAP family members, Livin binds directly to certain caspases via its baculoviral IAP repeat (BIR) domain and subsequently initiates their inactivation and degradation, thereby inhibiting cellular apoptosis (7-9). Several studies have documented that Livin mRNA and protein are undetectable in most adult human tissues, but are abundantly expressed in diversified types of human tumor tissues, including bladder cancer (10,11), non-small cell lung cancer (NSCLC) (12), nasopharyngeal carcinoma (13), renal cell carcinoma (14), hepatocellular carcinoma (15), neuroblastoma (16), colorectal cancer (17), melanoma (18) and gastric cancer (19). In addition, a series of functional studies have demonstrated that knockdown of Livin by RNA interference (RNAi) inhibits proliferation and invasion, promotes apoptosis and increases chemosensitivity in cancer cells (20-28). These findings highlight Livin as a promising therapeutic target for cancer prevention and treatment (6). Recently, Nedelcu *et al* have reported that Livin is frequently overexpressed in osteosarcoma, and overexpression of Livin

is associated with poor patient survival (29). To our knowledge, however, the functional role of Livin in osteosarcoma remains to be elucidated.

In the present study, we used an RNAi-based approach specifically targeting Livin mRNA to investigate how stable depletion of Livin affects the malignant phenotypes of human osteosarcoma cells. We report that suppression of endogenous Livin levels in human osteosarcoma cells decreased cell proliferation, migration and invasion, and also increased chemosensitivity. Our data suggest that Livin contributes the aggressive behavior of osteosarcoma and may serve as a potential target for osteosarcoma therapy.

Materials and methods

Cell culture. The human osteosarcoma cell lines MG-63, U2-OS and Saos-2 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air. For passaging, all cells were periodically detached with 0.25% trypsin-ethylene diamine tetraacetic acid solution and subsequently replated. Cells in the logarithmic phase of growth were used for the experiments.

Plasmid construction. The pGCsi-H1 plasmid (GeneChem, Shanghai, China) was used to generate short hairpin RNA (shRNA) specific for Livin. A pair of DNA template oligonucleotides encoding the target sequence of Livin mRNA (GeneBank accession: NM_022161.2) was designed and chemically synthesized as follows: sense, 5'-GATCCCC*AGTGGTTCCCCAGCTGTCA*TTCAAGAGA*TGACAGCTGGGGAACCACT*TTTTT-3'; antisense, 5'-AGCTAAAAA*AGTGGTTCCCCAGCTGTCA*TCTCTTGAA*TGACAGCTGGGAACCACT*GGG-3'. The italic sequences indicate insert sequences targeting Livin mRNA (519-537 bp). These oligonucleotides were annealed and subcloned into the *Hin*dIII and *Bam*HI sites of the pGCsi-H1 vector. Scrambled shRNA sequence with minimal homology to known mammalian genes was used as a negative control. The accuracy of the recombinant vectors was confirmed by restriction enzyme analysis and sequencing.

Transfection and selection for stable transfectants. For transfection, U2-OS cells were seeded into 6-well plates at a density of $1.0x10^5$ per well and allowed overnight growth to reach 70-80% confluence. Cells were then transfected with pGCsi-H1-Livin shRNA or control plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the supplier's protocol. At 48 h post-transfection, stable cell clones were obtained by Geneticin (G418, 800 μg/ml, Invitrogen) selection for 4 weeks. The individual G418-resistant clones were picked, identified for Livin expression, and maintained in medium containing 350 μg/ml G418 for further experiments.

Quantitative real-time PCR. Total RNA was isolated from treated cells with TRIzol reagent (Invitrogen) and reverse-transcribed to synthesize cDNA using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) according to the manufacturer's instructions. cDNA was subsequently amplified by SYBR-Green based real-time PCR on an Exicycler™ 96 thermal block (Bioneer, Daejeon, Korea) using the following primers: Livin, forward, 5'-GTCAGTTCCTGCTCCGGTCAA-3'; reverse, 5'-GGCTGCGTCTTCCGGTTCTT-3'; β-actin, forward, 5'-CTTAGTTGCGTTACACCCTTTCTTG-3'; reverse, 5'-CTGTCACCTTCACCGTTCCAGTTT-3'. After 10 min at 95˚C to activate DNA polymerase, the cycling parameters were as follows: 40 cycles consisting of denaturation at 95˚C for 5 sec, annealing at 60˚C for 30 sec, and extension at 72˚C for 30 sec. Primer specificity was confirmed by melting curve analysis. Livin mRNA expression levels were calculated by the comparative cycle threshold (Ct) method using β-actin as an internal reference for normalization (30).

Western blot analysis. Western blot analysis was performed using whole cell lysates, separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) as described previously (31). The membranes were immunoblotted overnight at 4˚C with rabbit polyclonal to Livin (1:500 diluted, ab97350), rabbit polyclonal to Bcl-2 (1:1,000 diluted, ab18210), rabbit polyclonal to Bax (1:1,000 diluted, ab7977), rabbit polyclonal to active Caspase-3 (1:200 diluted, ab2302), rabbit polyclonal to cleaved poly (ADP-ribose) polymerase (PARP) (1:1,000 diluted, ab4830), rabbit polyclonal to Cyclin D1 (1:200 diluted, ab95281), rabbit polyclonal to matrix metalloproteinase-2 (MMP-2) (1:500 diluted, ab53771), rabbit polyclonal to MMP-9 (1:1,000 diluted, ab38898) (all from Abcam, Cambridge, MA, USA), and mouse monoclonal to β-actin (1:5,000 diluted, Sigma-Aldrich, St. Louis, MO, USA). The membranes were then developed with horseradish peroxidase-labeled anti-mouse or anti-rabbit IgG by chemiluminescence substrate (Millipore). Protein expression levels were quantified by densitometric analysis.

Colony formation assay. The tested cells were seeded at $2x10^2$ cells per 10 mm dish and incubated at 37˚C for 10-14 days to allow colonies to form. The cell colonies were washed twice with phosphate-buffered saline (PBS), fixed with methanol, and stained with Giemsa staining solution. Visible colonies were counted manually.

Cell proliferation assay. Cell viability was evaluated by colorimetric assay using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). Briefly, cells were seeded into 96-well plates at a density of $5x10^3$ and allowed to adhere. After incubation for various time intervals, cells were stained with 100 μl sterile MTT dye (0.5 mg/ml, Sigma-Aldrich) for 4 h at 37˚C. Then, the supernatant was removed, and the resultant formazan crystals were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich). The absorbance value was read at 570 nm using a microplate reader.

Hoechst 33258 staining. The nuclear changes and apoptotic body formation were visualized with Hoechst 33258 staining. Cells were fixed with 4% paraformaldehyde at 4˚C

for 20 min, washed with PBS, and incubated with Hoechst 33258 (Beyotime Institute of Biotechnology, Haimen, China) at room temperature for 30 min. Finally, cells were mounted and examined by fluorescence microscopy.

Apoptosis and cell cycle analysis by flow cytometry. For cell cycle analysis, cells were detached by trypsin digestion, fixed with ice-cold 70% ethanol overnight, and stained with 50 μg/ml propidium iodide (PI) in the presence of RNase A at 37˚C for 30 min. Intracellular DNA content was analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Apoptotic cells were quantified by flow cytometry using an Annexin V-FITC/PI Apoptosis Detection Kit (KeyGen Biotech, Nanjing, China) according to the manufacturer's instructions. In brief, subconfluent culture of U2-OS cells or cells treated with 10 μg/ml cisplatin (Meilun Biotechnology, Dalian, China) for 24 h were harvested by trypsinization, washed with ice-cold PBS, and incubated with 500 μl of the Annexin V-binding buffer. Thereafter, the samples were stained with 5 μl of fluorescein isothiocyanate (FITC)-labeled Annexin V and 5 μl of PI at room temperature for 15 min in the dark before analysis by flow cytometry.

Wound healing assay. Cells were subcultured in 6-well plates at a density of $5x10^5$ cells/well and allowed to form a confluent monolayer. After removal of the culture medium, cell monolayers were gently scratched with a 200-μl pipette tip to create a linear wound. The wounded monolayers were washed twice with serum-free medium to remove cell debris, and cells were then allowed to migrate into the cell-free area. The scratch area was photographed immediately and at 12 and 24 h after scratching. Cell migration was calculated as the mean percentage of the cell migrated distance compared with the initial wound distance.

Matrigel invasion assay. Invasion of U2-OS cells was assessed using Transwell cell culture chambers (8 μm pore size, Costar, Cambridge, MA, USA) containing a Matrigel-coated polycarbonate membrane filter. The upper surface of the filter was coated with Matrigel (1 mg/ml, BD Biosciences). Cells ($1x10^4$ cells/well) in 500 μl of serum-free medium were placed in the upper chamber of the Transwell insert, and the lower chamber was filled with 750 μl culture medium with 10% FBS (chemoattractant). After incubation at 37˚C for 24 h, non-invasive cells on the upper surface of the filter were removed using a cotton swab, and the invasive cells on the bottom side of the membrane were fixed with formaldehyde and stained with hematoxylin. The number of cells was counted under the microscope from six random fields at a x200 magnification.

Statistical analysis. All experiments were repeated in triplicate and data are expressed as mean ± standard deviation (SD). Statistical differences between groups were evaluated by one-way analysis of variance, and the Bonferroni post hoc test was used for multiple comparisons. Graphs were plotted and statistical calculations were performed using SigmaPlot 12.0 (Systat Software Inc., San Jose, CA, USA). A P-value of <0.05 was considered statistically significant.

Results

Stable knockdown of Livin by plasmid-mediated shRNA in U2-OS cells. We first examined the expression of Livin protein by western blot analysis in several osteosarcoma cell lines (MG-63, U2-OS and Saos-2). As shown in Fig. 1A, relative high expression of Livin protein was observed in all analyzed cell lines. To further characterize the biological role of Livin in osteosarcoma, we employed an RNAi approach to knockdown Livin expression in U2-OS cells expressing the highest level of endogenous Livin. Stable transformants were obtained by G418 selection and identified by quantitative real-time PCR and western blot analysis. The results showed that the Livin mRNA and protein levels in cells transfected with specific shRNA for Livin were significantly reduced by 70 and 73%, respectively, compared with those transfected with control shRNA ($P<0.01$, Fig. 1B and C). Thus, the specific shRNA targeting Livin mRNA could effectively knockdown Livin expression at both transcriptional and translational levels.

Stable knockdown of Livin expression decreased cell proliferation and in vitro tumorigenic potential of U2-OS cells. Next, we evaluated the effects of shRNA-mediated stable knockdown of Livin expression on anchorage-independent growth by the colony formation assay. Compared with U2-OS cells expressing control shRNA, U2-OS cells with Livin knockdown produced fewer and smaller colonies, suggesting a reduced *in vitro* tumorigenic potential ($P<0.01$, Fig. 2A). In addition, the effects of Livin knockdown on cell proliferation was also assessed by the MTT assay. As illustrated in Fig. 2B, suppression of Livin resulted in a significant decrease in the proliferation rate of U2-OS cells at 48, 72 and 96 h ($P<0.01$). Together, our data indicate that Livin contributes to cell proliferation and *in vitro* tumorigenic potential of U2-OS cells.

Stable knockdown of Livin expression induced apoptosis and cell cycle G1-S phase arrest in U2-OS cells in vitro. To explore the possible mechanisms underlying the actions of Livin on the growth of U2-OS cells, we evaluated the cell cycle distribution in Livin shRNA transfected cells and control cells by using flow cytometry analysis. Fig. 3A shows that Livin depletion in U2-OS cells caused an accumulation of cells in the G0/G1 phase and a decrease in the S phase and G2/M phase as compared with control shRNA transfected cells. Consistently, a significant decreased level of cyclin D1 was detected in Livin shRNA transfected cells ($P<0.01$, Fig. 3B). These data suggest that downregulation of Livin arrests cell cycle at the G0/G1 phase, thereby regulating osteosarcoma cell growth.

Hoechst nuclear staining and flow cytometry analysis were undertaken to investigate the effects of Livin shRNA on apoptosis *in vitro*. Representative photographs of cells stained with Hoechst are shown in Fig. 4A. The formation of typical apoptotic bodies was observed in U2-OS cells with knockdown of Livin, but not in the control cells. Results from Annexin V-FITC/PI analysis also revealed that U2-OS cells transfected with Livin shRNA (31.86±8.16%) underwent obvious apoptosis than control shRNA transfected cells (4.34±1.55%) and untransfected cells (4.83±1.15%, Fig. 4B). We also analyzed protein expression levels of several apoptosis-associated factors by western blot analysis. Compared with control cells, the protein

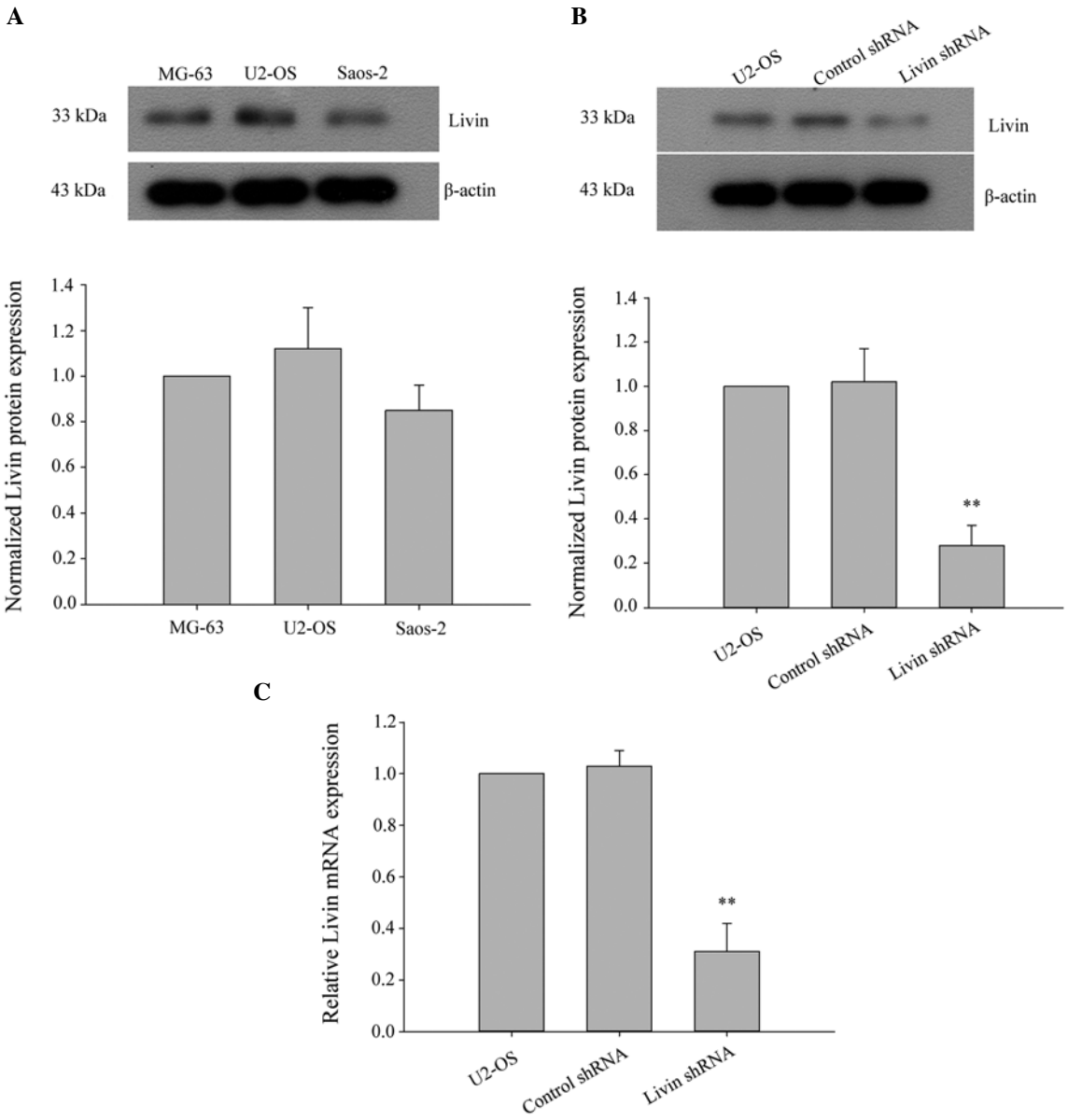

Figure 1. shRNA-mediated inhibition of Livin protein expression in osteosarcoma cells. (A) Western blot analysis of Livin in a panel of osteosarcoma cell lines. Specific gene silencing of Livin appeared in shRNA-transfected U2-OS cells by (B) western blot analysis and (C) quantitative real-time PCR. Representative blots are shown and protein size is expressed in kDa. β-actin was used as a loading control. Data are expressed as means ± SD. **P<0.01 compared with control shRNA cells.

expression levels of activate Caspase-3, Bax and cleaved PARP were significantly increased, whereas the expression level of Bcl-2 was remarkably reduced in Livin shRNA transfected U2-OS cells (P<0.05, Fig. 4C). Collectively, our observations suggest that suppression of cell growth by Livin shRNA is partially attributable to increased apoptosis *in vitro*.

Stable knockdown of Livin expression inhibited migration and invasion capacity of U2-OS cells in vitro. Livin has been shown to be required for migration and invasion of several types of cancer cells (25,27,32). Therefore, we determined the effects of Livin depletion on migration and invasion ability of U2-OS cells using the wound healing assay and the Transwell invasion assay, respectively. As shown in Fig. 5A, the wound healing capacity was obviously reduced in stably Livin knockdown cells as compared with control cells. Moreover, the Transwell invasion assay showed that Livin depletion significantly suppressed invasion of U2-OS cells *in vitro*, as evidenced by a significant decrease in the number of cells that invaded through the Matrigel-coated membrane (P<0.05, Fig. 5B). To investigate the mechanism responsible for the reduced migration and invasion ability caused by downregulation of Livin, we quantified the protein expression levels of MMP-2 and MMP-9 by western blot analysis. In comparison to U2-OS cells and control shRNA transfected cells, MMP-2 and MMP-9 protein levels were significantly reduced in stably Livin knockdown cells (P<0.01, Fig. 5C). Altogether, the results imply that knockdown of Livin reduces the metastatic ability of osteosarcoma cells by downregulating MMP-2 and MMP-9.

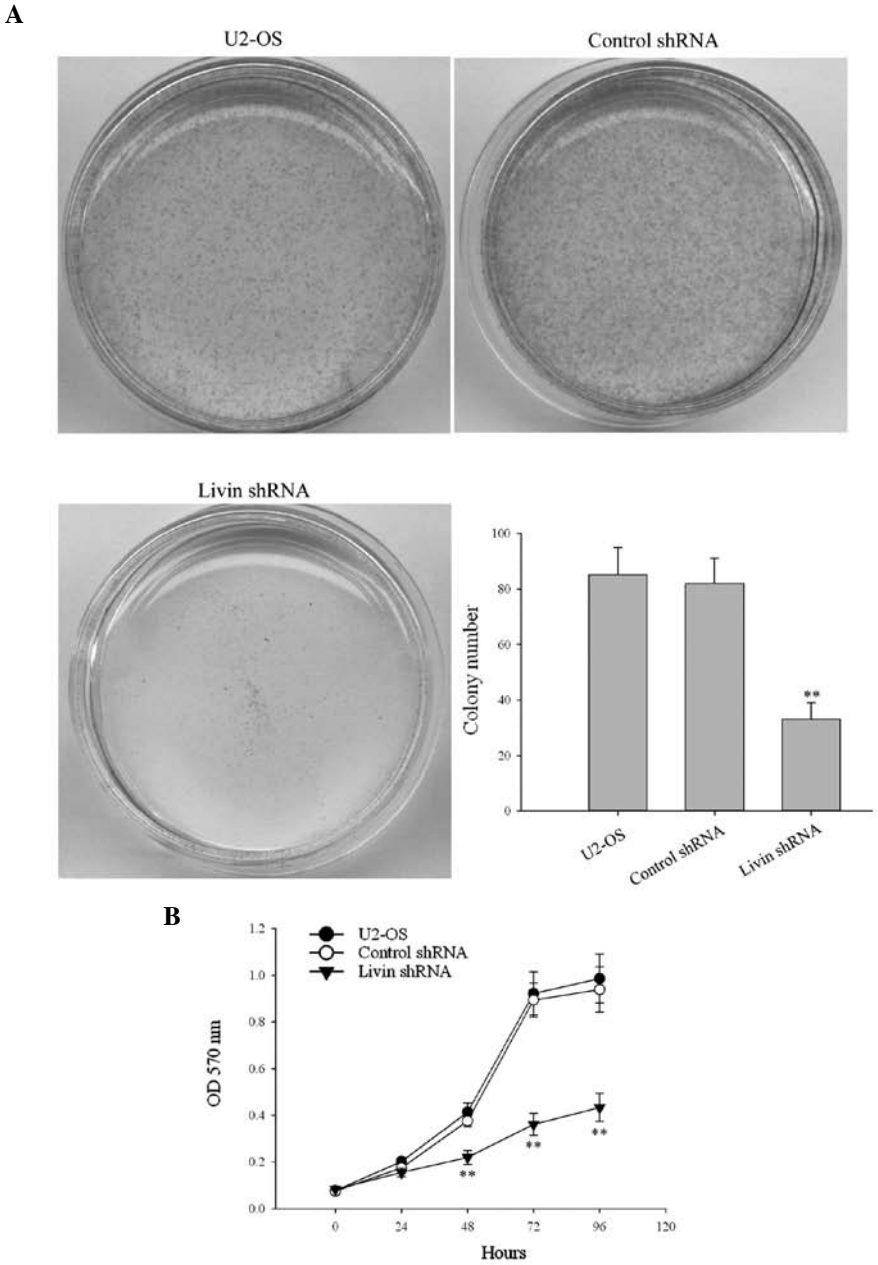

Figure 2. Stable knockdown of Livin inhibited cell proliferation and anchorage-independent growth of U2-OS cells *in vitro*. (A) In colony formation assay, U2-OS cells were seeded at a density of $2x10^2$ cells per 10 mm dish, and colonies were allowed to grow for 10-14 days. Positive colonies were counted manually. (B) For the MTT assay, U2-OS cells were seeded into 96-well plates and incubated for different time periods. The experiments were performed in triplicate. $^{**}P<0.01$ compared with control shRNA cells.

Stable knockdown of Livin expression enhanced cisplatin cytotoxicity in vitro. Previous studies have documented that depletion of Livin by RNAi increases apoptosis caused by cisplatin in lung (33), gastric (21) and colon (34) cancer cell lines. To test whether knockdown of Livin in U2-OS cells affects their sensitivity to cisplatin, Livin shRNA transfected cells and control cells were treated with 10 μg/ml cisplatin for 24 h and analyzed for apoptosis by flow cytometry. As indicated in Fig. 6, depletion of Livin in U2-OS cells significantly increased cell death after treatment with cisplatin (66.61±9.28%) compared with control cells (41.71±6.55% and 42.44±7.13%, P<0.01). These findings indicate that Livin plays a crucial role in modulating cisplatin cytotoxicity *in vitro*.

Discussion

A retrospective study has shown that high expression of Livin protein in tumor tissues predicts poor survival for patients with osteosarcoma (29), but the potential roles of Livin in osteosarcoma cell growth, migration and drug toxicity have not been investigated. In the present study, we employed a vector-based RNAi strategy to stably silence endogenous Livin expression in U2-OS osteosarcoma cells. Quantitative real-time PCR and western blot analysis confirmed that the pGCsi-H1-Livin shRNA vector was able to dramatically abolish the Livin expression in U2-OS cells. Our further experiments demonstrated that knockdown of

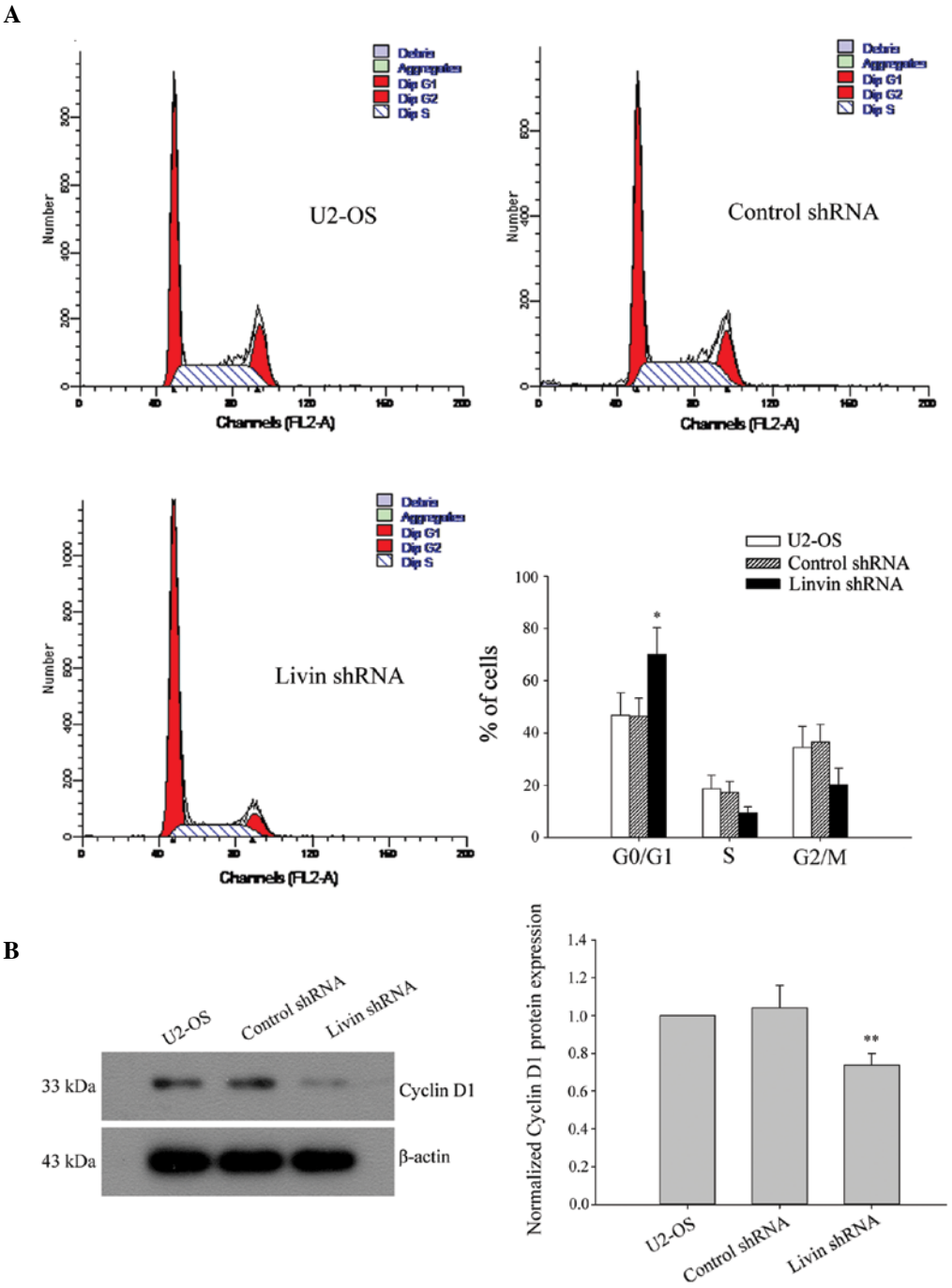

Figure 3. Stable knockdown of Livin induced cell cycle arrest at the G0/G1 phase and downregulated Cyclin D1 in U2-OS cells. (A) Cells at 80% confluence were stained with PI and quantified for the cycle distribution by flow cytometry. Data are presented as averages from three independent experiments. (B) Western blot analysis of Cyclin D1 protein expression in stably transfected cells. Representative blots are shown, and quantitative data are expressed as the intensity ratio of Cyclin D1 to β-actin. *P<0.05, **P<0.01 compared with control shRNA cells.

Livin in U2-OS cells significantly inhibited cell proliferation and colony formation *in vitro*. In addition, flow cytometry analysis showed that depletion of Livin reduced the number of cells in the S phase and G2/M phase, while increased the number of cells in the G0/G1 phase, indicating G0/G1 cell cycle arrest. These data are consistent with the published findings in other tumor cells (26,35-37). Cyclin D1 is a key regulator of the G0/G1 to S phase transition. Recently, Chen *et al* have demonstrated that intratumoral injection of lentivirus-delivered Livin shRNA into SPC-A1 lung cancer xenografts resulted in dramatic silencing of Livin, reduction in tumor growth, and downregulation of Cyclin D1 (38). In this study, our *in vitro* data consistently indicated that knockdown of Livin in U2-OS cells significantly reduced the expression level of Cyclin D1. Furthermore, it is also reported that knockdown of Livin can induce the cancer cell cycle arrested at the G0/G1 phase and finally lead to apoptosis (26,35). Our present experiments showed that reduced expression of Livin significantly induced apoptotic cell death in U2-OS cells. In corroboration with these results,

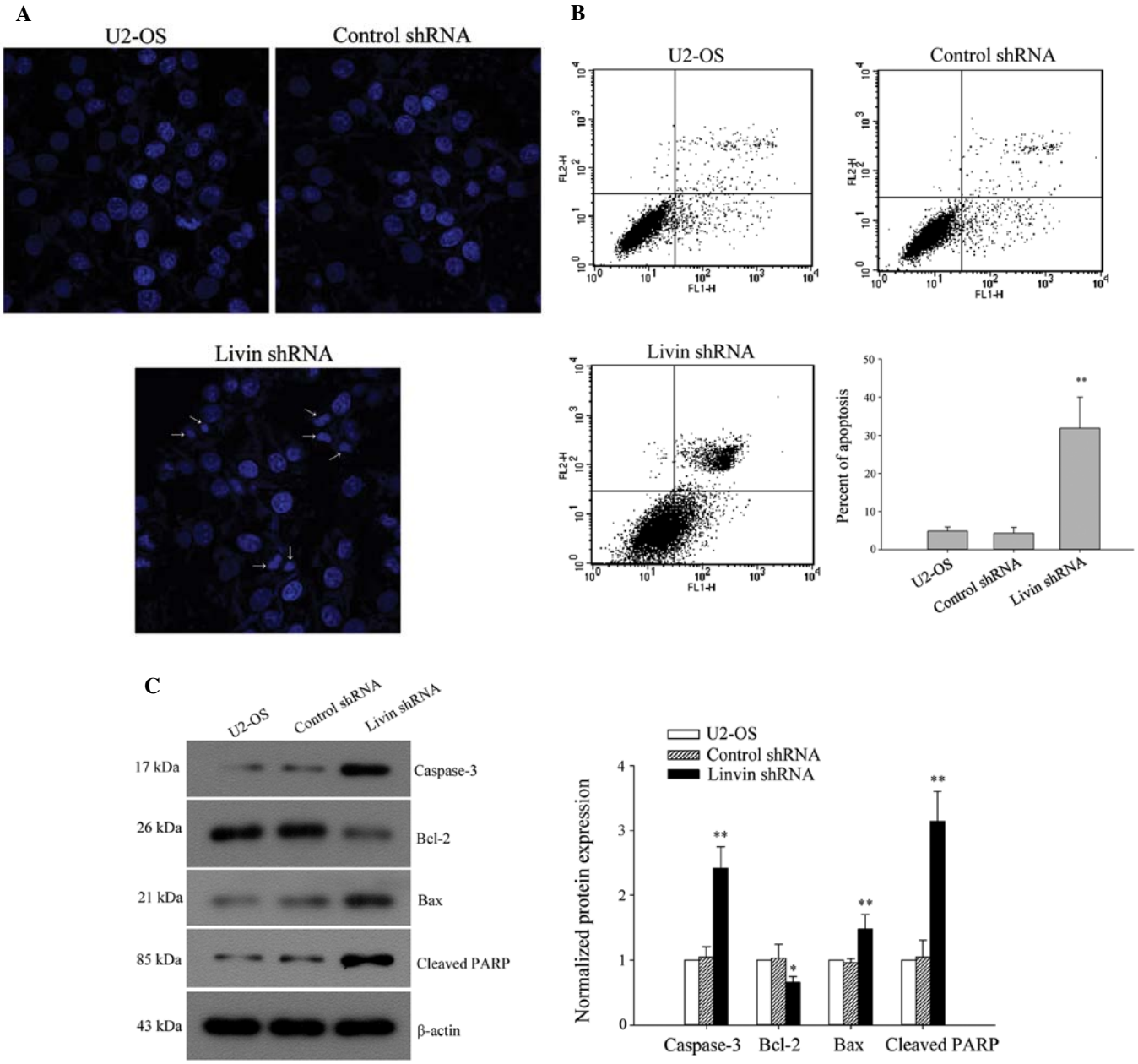

Figure 4. Stable knockdown of Livin induced apoptosis in U2-OS cells. (A) Hoechst 33258 staining showing apoptotic morphological changes in U2-OS cells. Cells were stained with Hoechst 33258 and examined by fluorescence microscopy. Arrows denote typical apoptotic cells. (B) Flow cytometry analysis with Annexin V-PI staining was performed to evaluate the percentage of apoptotic cells. The number of PI/Annexin V single positive and Annexin V/PI double positive cells was calculated as the measurement of apoptotic cells. Data are expressed as means ± SD. (C) Western blot analysis of activate Caspase-3, Bax, Bcl-2 and cleaved PARP protein levels in U2-OS cells. Densitometric values were normalized by β-actin. *P<0.05, **P<0.01 compared with control shRNA cells.

stably knockdown of Livin resulted in the elevation of activate Caspase-3, Bax and cleaved PARP as well as reduced Bcl-2 levels in U2-OS cells. Taken together, these findings generally support the mechanisms to explain how silencing Livin leads to proliferation inhibition of osteosarcoma cells. Nonetheless, the precise mechanisms by which knockdown of Livin causes cancer cell growth inhibition and cell cycle arrest need to be further elucidated.

Invasive and metastatic capacities are important malignant properties of cancer cells. Several lines of evidence have demonstrated that Livin plays a critical role in acquisition of invasiveness of cancer cells (27,32). In the present study, the wound healing assay and the Transwell invasion assay were conducted to determine the effects of Livin depletion on migration and invasion ability of U2-OS cells. The results showed that knockdown of Livin significantly reduced the invasive and metastatic ability of osteosarcoma cell *in vitro*. Proteolytic degradation of the basement membrane and extracellular matrix (ECM) by MMPs is an initial step for tumor invasion and metastasis. In particular, MMP-2 and MMP-9 are consistently expressed in malignant tissues and associated increased invasiveness in various cancer cells (39). The present study demonstrated a greater reduction in MMP-2 and MMP-9 protein levels in Livin knockdown U2-OS cells, with parallel results in the wound healing assay and the Transwell invasion assay. Although there is a lack of

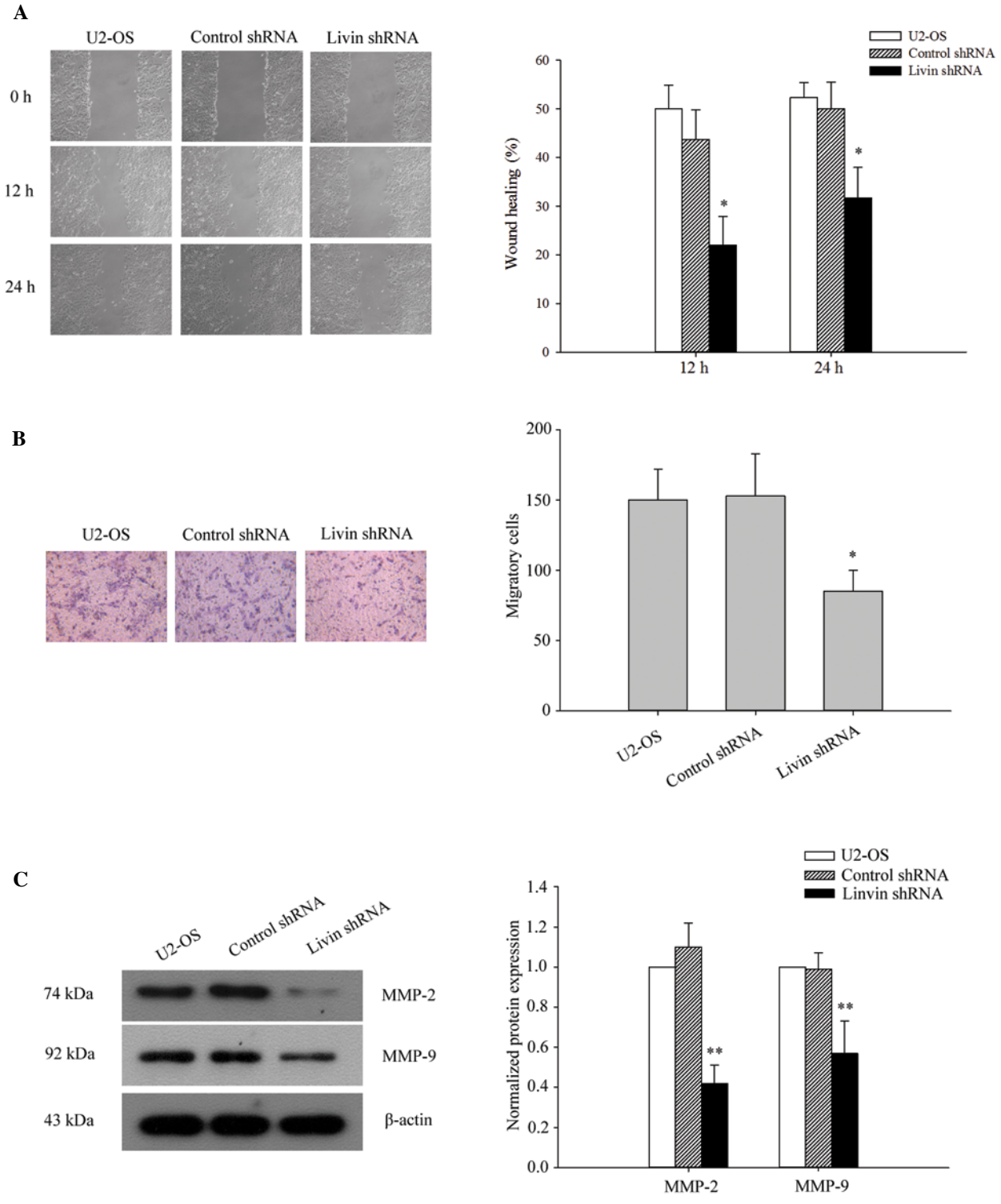

Figure 5. Stable knockdown of Livin suppressed osteosarcoma U2-OS cell migration and invasion *in vitro*. (A) Wound healing assay of the cell migration. Cell monolayers were scratched with a sterile 200-μl pipette tip and images were taken at the indicated time points. Bars represent as the mean percentages of the remaining cell-free area compared with the area of the initial wound. (B) Transwell migration assay of cell invasion. The same number of cells was seeded on the Transwell. After 24 h, the number of cells that had migrated to the lower chamber was counted. Values are expressed as means ± SD of three independent experiments. (C) Western blot analysis of MMP-2 and MMP-9 protein expression levels in stably transfected cells. The blots shown are representative of three independent experiments. Columns represent means ± SD. *P<0.05, **P<0.01 compared with control shRNA cells.

direct evidence, our data suggest that knockdown of Livin reduces the metastatic ability of osteosarcoma cell by down-regulating MMP-2 and MMP-9. Additionally, Livin has been reported to promote prostate cancer cell invasion via the nuclear factor-κB (NF-κB) signaling pathway (27,32). NF-κB is a key transcriptional factor for the production of MMP-2 and MMP-9 (40). Therefore, it seems plausible that down-regulation of MMP-2 and MMP-9 by Livin knockdown may

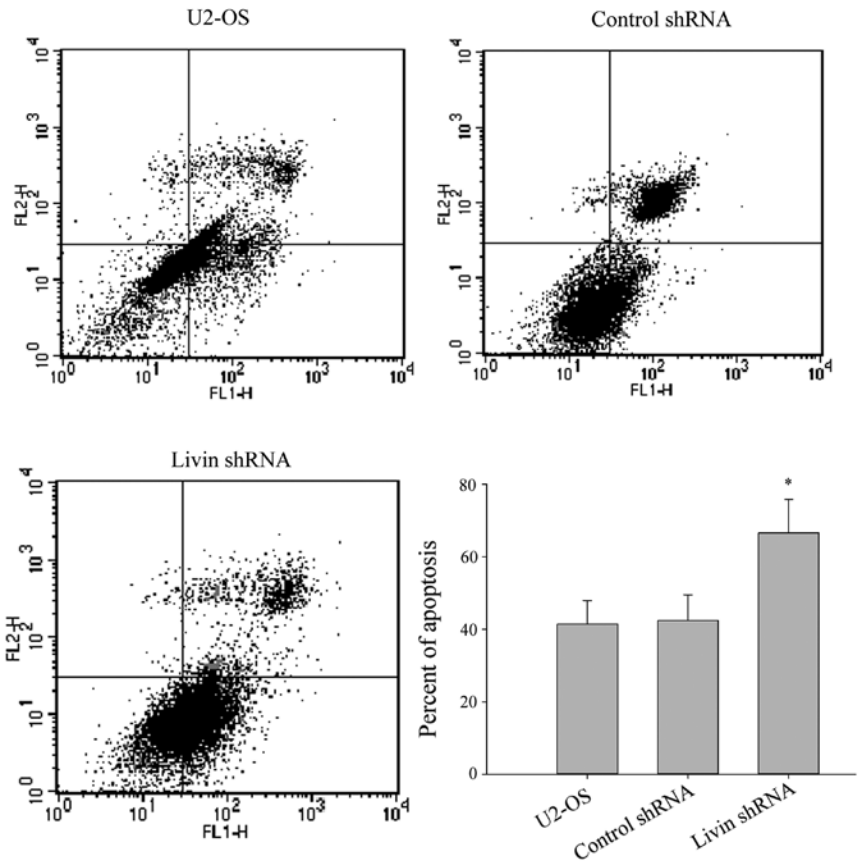

Figure 6. Stable knockdown of Livin increased chemosensitivity to cisplatin in U2-OS cells. Cells were treated with 10 μg/ml cisplatin for 24 h and analyzed for apoptosis by flow cytometry with Annexin V-PI staining. Bars represent the percentages of apoptosis from three independent experiments. *P<0.05 compared with control shRNA cells.

be also mediated via the NF-κB signaling pathway. However, this assumption requires further investigation.

Cumulative evidence has suggested a novel role of Livin in induction of resistance to various chemotherapeutic drugs, such as cisplatin, vincristine (VCR), etoposide (VP-16) and 5-flourouracil (5-FU) (23,34). Osteosarcoma U2-OS cells are notorious for their resistance to conventional chemotherapeutic regiments (41). To date, however, whether Livin contributes to chemoresistance in osteosarcoma cells has not been investigated. In the current study, we showed that Livin downregulation in U2-OS cells remarkably enhanced apoptosis in response to cisplatin treatment, suggesting that Livin inhibition sensitizes tumor cells with Livin levels that show a high degree of chemoresistance. This is in agreement with previous studies indicating that Livin plays an important role in chemoresistance in various cancer cell lines (20-21,33). Our data, together with previous findings, suggest that targeting of Livin by RNAi combined with chemotherapy provides a potential synergistic therapy for patients with osteosarcoma.

In summary, the present study demonstrated that knockdown of Livin by shRNA effectively inhibited cell growth and invasion, arrested cell cycle at the G0/G1 phase, promoted spontaneous apoptosis, and increased chemosensitivity to cisplatin in osteosarcoma cells. Our findings suggest that Livin may serve as a promising therapeutic target for osteosarcoma.

Acknowledgements

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