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Original Article

CKB affects human osteosarcoma progression by regulating the p53 pathway

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Abstract: This study aimed to explore the role of the creatine kinase B (CKB) gene in the development of human osteosarcoma (OS). Western blotting and qRT-PCR were performed to detect CKB expression in tissues and cells. CCK-8, colony formation, flow cytometry, Transwell, and cell scratch assays were performed to detect OS cell viability, proliferation, apoptosis, invasion, and migration. Gene set enrichment analysis (GSEA) was used to conduct signal pathway enrichment. CKB expression was higher in OS tissues and cells than that in normal tissues and cells. Silencing CKB expression reduced cell proliferation, migration, and invasion, and improved cell apoptosis in HOS cells, while overexpressing CKB increased cell proliferation, migration, and invasion, and decreased apoptosis in U2-OS cells. GSEA showed that CKB affected the p53 signaling pathway. Overexpression of CKB inhibited the protein expression of p53, p21, and Bax and promoted the expression of Bcl-2 and MDM2 in U2-OS cells. Conversely, silencing CKB promoted the protein expression of p53, p21, and Bax, and inhibited the expression of Bcl-2 and MDM2 in HOS cells. Silencing p53 could reverse the effect of the silencing CKB in HOS cells, and overexpressing p53 could reverse the effect of overexpressing CKB in U2-OS cells. Taken together, CKB affects the development of OS by regulating the activity of the p53 signaling pathway.

Keywords: Osteosarcoma, p53 signaling pathway, CKB, cell proliferation, cell invasion

Introduction

Osteosarcoma (OS) is a malignant bone cancer, which has very high incidence and a poor prognosis [1, 2]. The incidence of OS in children and adolescents is higher than that in other age groups [3]. The traditional treatment methods for OS include surgical resection and adjuvant chemotherapy [4]. However, surgical resection results in bone loss at the resection site [5], which affects the patient's prognosis. Therefore, halting the progression of OS is a hot topic in current scientific research. Currently, in addition to clinical surgical resection, gene therapy has important practical significance in halting the progression of OS.

Creatine kinase B (CKB) is a cytoplasmic subtype of creatine kinase, and its expression is upregulated in many cancers. A previous study reported that knockdown of CKB led to cytoskeleton damage, thereby reducing the migration of human endometrial cancer cells (Ishikawa) [6]. MiR-551a and miR-483-5p can significantly reduce colon cancer metastasis by inhibiting CKB [7]. Macrophages play a role in tumor angiogenesis and inflammatory reactions [8]. Ma et al. reported that BspJ regulated macrophage apoptosis through the interaction with NME2 and CKB [9]. The increase in the level of CKB methylation influences the occurrence of gastric cancer, and CKB expression or its DNA methylation may be a useful marker for predicting tumor progression and developing targeted anti-cancer therapies [10].

P53 signaling plays a very important role in regulating cell apoptosis. P53 mutations are a common cause of cancer [11]. In a previous study, it was found that CYZ2017, a protein inhibitor, could cause cellular p53 levels to increases, and thus induce p21 activation, which results in G0/G1 cell cycle hysteresis and apoptosis in HCT116 cells [12]. Methoxy eugenol can increase activity in the p53 pathway and increase the expression of p53 and p21 to

inhibit cell proliferation, thereby arresting the development of endometrial cancer [13]. The upregulation of miR-377 promotes the activity of the p53/p21 pathway, thereby inhibiting the self-renewal of esophageal squamous cell carcinoma (ESCC) cells and inhibiting the development of cancer [14]. Arbutin can induce p53 expression at a certain concentration, increase the ratio of Bax/Bcl-2, and therefore promote apoptosis in prostaglandin cells [15]. Knockdown of IRX5 decreases Bcl-2 expression and increases the expression of p53 and Bax, thus promoting hepatocellular carcinoma cell apoptosis [16]. The p53 signaling pathway has also been found to play a role in OS. Combination therapy with radiation and cisplatin could increase p53 expression in MG-63 cells, thereby increasing the rate of apoptosis in MG-63 cells and causing G2 phase hysteresis [17].

The characteristics of tumor development include uncontrollable cell proliferation and strong migration and invasion abilities. Metalloproteinases (MMPs) are involved in the process of cell migration and invasion. The secretion of MMPs is crucial for the metastasis of cancer cells because MMPs are responsible for degradation of the extracellular matrix [18]. A previous study reported that propiolactone could regulate Bax, Bcl-2, and p53 to induce apoptosis of gastric cancer cells and regulate MMP-2, MMP-7, and MMP-9 to inhibit gastric cancer cell migration and invasion [19]. MMP-7 was overexpressed in nine cases of invasive ductal pancreatic adenocarcinoma and seven cases of invasive intraductal papillary mucinous adenocarcinoma [20], indicating that there is a link between MMPs and tumors.

However, the role of CKB in OS remains unknown. We detected CKB expression in OS tissues and cells, and investigated whether overexpression and knockdown of CKB were related to OS cell growth, apoptosis, and invasion.

Material and methods

Patients and clinical tissue specimens

Twenty-two pairs of OS tissues and corresponding non-cancerous tissues were collected from our hospital. The patients in this study did not receive chemotherapy or radiotherapy before surgery. All samples were processed and anonymized in accordance with ethical and legal standards. Written informed consent was obtained from every patient, and the ethics committee of our hospital approved the study.

Cell culture

The normal human osteoblast immortalized cell line hFOB 1.19 and OS cells (U2-OS, MG-63, SAOS-2, and HOS) were purchased from the Procell Life Science & Technology Co., Ltd (Wuhan, China). High-glucose Dulbecco's modified Eagle medium (DMEM) with 10% foetal bovine serum (FBS) was used to maintain the cells, and the cells were cultured aseptically at 37°C with 5% CO_2.

Cell transfection

U2-OS and HOS cells (3×10^4 cells/ml) were plated in the six-well plates. A total of 7.5 μl Lipofectamine 2000 (Invitrogen, USA), 117.5 μl plasmid incubation solution opti-MEM (Gibco, USA), and 10 μl pcDNA3.1-CKB, pcDNA3.1-p53, siCKB-1, siCKB-2, si-p53, pcDNA3.1 vector or scrambled si-NC were added into each well. The pcDNA3.1 vector, pcDNA3.1-CKB, pcDNA3.1-p53, si-NC, siCKB-1, siCKB-2, and si-p53 were synthesized by Shanghai GenePharma Co., Ltd (Shanghai, China). Cells were used for subsequent experiments after transfection for 48 h.

Western blotting

Proteins were extracted from the cells and tissues using RIPA buffer (Sigma-Aldrich), and the entire procedure was performed on ice to prevent protein degradation. Samples of equal amounts (30 μg) were added to the protein loading buffer. The concentrating gel and separation gel of SDS-PAGE were prepared, and the gel was spotted after it had solidified. A constant voltage (80 V) was used for electrophoresis. After electrophoresis, the proteins were transferred to a PVDF cellulose acetate film; 200 mA constant current was used for wet transfer. After blocking with skimmed milk powder for 1 h, the membranes were incubated with the protein primary antibody (CKB, ab108388, Abcam; GAPDH, 5174, Cell signaling technology; MMP-7, 3801, Cell Signaling Technology; MMP-9, 13667, Cell Signaling Technology; p53, 2527, Cell Signaling Technology; p21, ab109520, Abcam; Bax, 5023, Cell

Signaling Technology; Bcl-2, ab32124, Abcam; MDM2, 86934, Cell Signaling Technology) and secondary antibody, and the ultra-sensitive exposure solution was used for exposure imaging.

QRT-PCR assay

Total RNA was extracted from the cells and tissues. The reverse transcription kit was used to synthesize the template cDNA. A NanoDrop was used to determine the cDNA concentration, and fragment amplification was performed using the qPCR kit. The reaction system was composed of: 100 μl: template cDNA (10 μl), each primers (2 μl), SYBR Green1 dye (20 μl), ddH_2O (66 μl). The primer sequence (synthesized by Shanghai Shenggong Biological Co., Ltd., China) used in this study was as follows: CKB: F: 5'-TCACTCCCGTCTCGCCTC-3', R: 5'-CAGGCTGGACAGGGCTTC-3'; GAPDH (an internal reference gene): F: 5'-AGGTCGGAGTCAACGGATTT-3', R: 5'-TCGCCCCACTTGATTTTGGA-3'.

Cell scratch assay

After transfection, U2-OS and HOS cells (1×10^5) were seeded into 6-well plates. A 20 μl pipette tip was used to scratch a line on the bottom of the plate. At 0 and 24 h after inscribing, the scratch area was measured, and the cell migration rate was calculated.

CCK-8 assay

After transfection, U2-OS and HOS cells (3×10^3 cells/well) were seeded in 96-well plates. A total of 15 μl CCK-8 solution was slowly added to the wells after culturing for 24, 48, 72, and 96 h. Subsequently, cells were cultured for 3 h in a constant-temperature incubator. After incubation, the absorbance (450 nm) was measured using a microplate reader.

Colony formation assay

After transfection, HOS and U2-OS cells were cultured in DMEM with 10% FBS in a constant-temperature incubator. During colony formation, a single cell gradually proliferates into a small population of individual cells called cell colonies. When the cells to be cultured appeared as colonies, the cells were washed with PBS, fixed with methanol, and stained with Giemsa. Finally, the colonies were observed under a microscope and photographed.

Flow cytometry analysis

After transfection, HOS and U2-OS cells were collected and then resuspended in the binding buffer. Next, Annexin V and PI were used to treat the cells for 30 min at room temperature. Finally, the FACScan flow cytometer was used to measure cell apoptosis.

Transwell assay

After transfection, HOS and U2-OS cells were harvested and transferred to the upper chamber of the Transwell chamber containing Matrigel and cultured in serum-free DMEM. DMEM containing 10% FBS was added to the lower chamber of the Transwell. After culturing for 48 h, the cells on the top surface of the lower chamber were removed. Cells on the bottom of the lower chamber were fixed with formaldehyde and then stained with crystal violet. Finally, the cells were observed under a microscope and photographed.

Differentially expressed genes (DEGs) analysis

At the National Center for Biotechnology Information, the GSE42572 datasets were downloaded from Gene Expression Omnibus (GEO) database [21]. The detailed information for GSE42572 datasets can be reviewed at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42572. The DESeq2 R package was used to perform analysis of differential expression.

Data analysis

GraphPad Prism software was used to analyze the data and generate the corresponding histogram. The analysis of variance was performed using t-test and analysis of variance (ANOVA) followed by Tukey's post-hoc test. Statistical significance was set at $P < 0.05$.

Results

CKB is highly expressed in human OS

GEO data (GSE42572) analysis showed that CKB expression in mesenchymal stromal cells of patients with OS was higher than that in

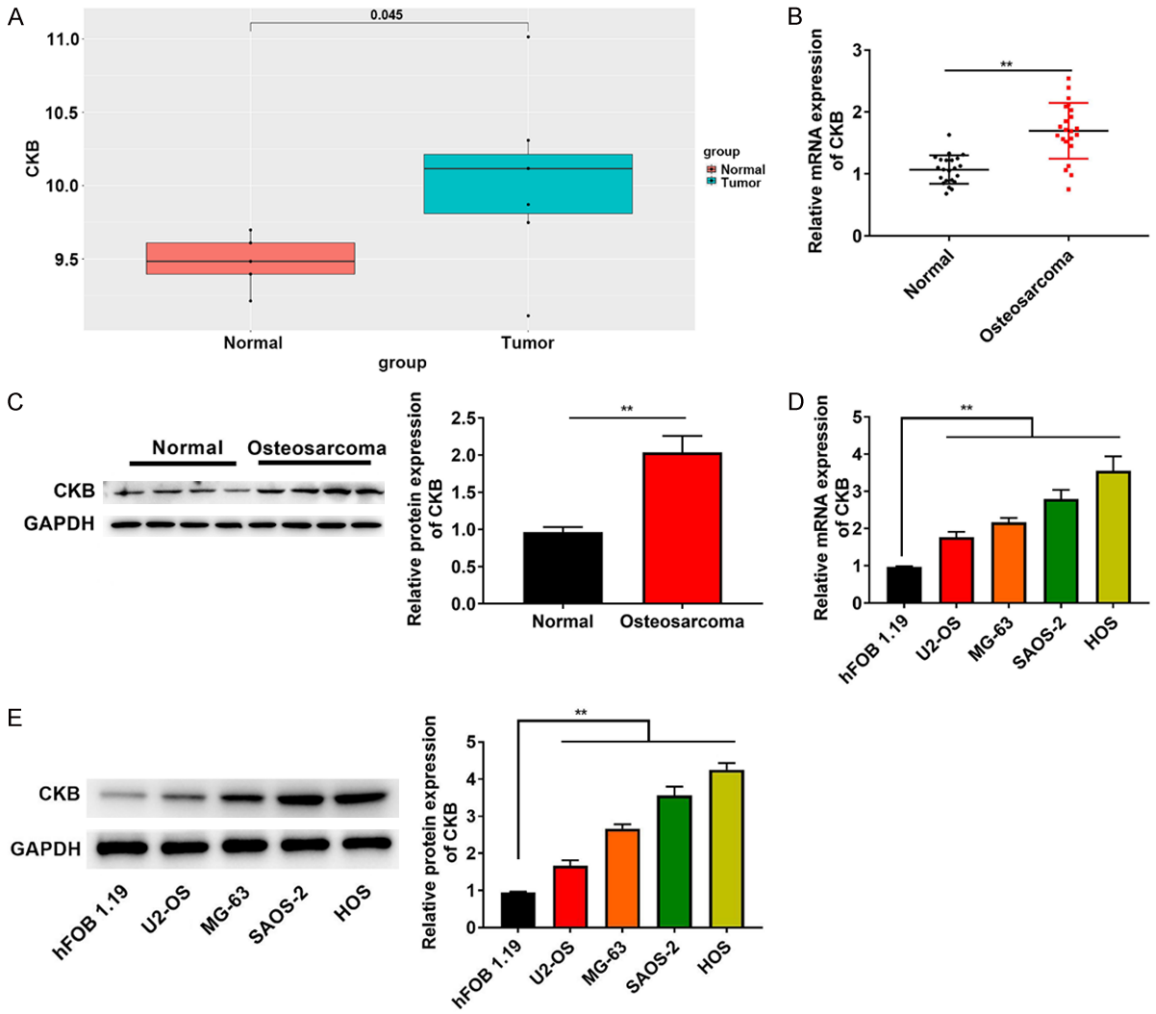


Figure 1. CKB was highly expressed in osteosarcoma (OS) tissues and cells. A. The GEO database (GSE42572) was used to analyze CKB expression in mesenchymal stromal cells of OS patients and healthy donors; B. QRT-PCR was used to detect CKB mRNA expression in 22 pairs of human OS tissues and adjacent normal tissues; C. CKB protein expression in human OS tissues and adjacent normal tissues was assessed using western blotting; D. QRT-PCR was used to detect CKB mRNA expression in human osteoblast immortalized cell line (hFOB 1.19) and OS cell line (U2-OS, MG-63, SAOS-2, and HOS). E. CKB expression in the human osteoblast immortalized cell line (hFOB 1.19) and OS cell line (U2-OS, MG-63, SAOS-2, and HOS) was assessed using western blotting. **P < 0.01.

healthy donors (**Figure 1A**; P = 0.045). To further verify the increased expression of CKB in OS, 22 pairs of OS tissues and normal tissue samples were collected to detect the changes of CKB levels. As shown in **Figure 1B**, the expression of CKB at the mRNA level in OS tissues was higher than that in the normal tissue (P < 0.01). In addition, the protein expression levels of CKB were also higher than those in normal tissues (**Figure 1C**, P < 0.01), suggesting that CKB has clinical significance in controlling OS. Next, the expression of CKB in OS cell lines (U2-OS, MG-63, SAOS-2, and HOS) and hFOB 1.19 cells was detected. As shown in **Figure 1D** and **1E**, compared with hFOB 1.19 cells, the expression levels of CKB in OS cells were higher (P < 0.01). Among OS cells, U2-OS cells had the lowest CKB expression, and HOS cells had the highest CKB expression, therefore we chose to use U2-OS and HOS cells in follow-up experiments.

The effect of CKB on the proliferation and apoptosis of OS cells

To further study the influence of CKB on OS, CKB plasmid was transfected into U2-OS cells, and two interference fragments of CKB were

transfected into HOS cells. Western blot and qRT-PCR assays were used to verify the transfection efficiency. The results showed that the levels of CKB in the CKB group were higher in U2-OS cells, while the levels of CKB in the siCKB-1 and siCKB-2 group were lower in HOS cells (**Figure 2A** and **2B**). This indicated that the transfection was successful. Compared with the Vector group, the cell proliferation in U2-OS cells was enhanced after overexpression of CKB (**Figure 2C**, $P < 0.01$), while CKB silencing in HOS cells decreased cell proliferation. Colony formation assay also confirmed this result (**Figure 2D**). Hydroxyurea (HU), a recommended anti-cancer medicine, has been utilized to treat various tumors. In our study, we used HU to induce apoptosis and detected the apoptosis rate using flow cytometry. HU increased apoptosis in HOS and U2-OS cells (**Figure 2E**, $P < 0.01$). Overexpression of CKB decreased the apoptosis induced by HU in U2-OS cells, while silencing of CKB expression further increased the apoptosis induced by HU in HOS cells.

Effect of CKB on the migration and invasion of OS cells

Overexpression of CKB increased the wound healing rate of U2-OS cells, while silencing CKB reduced the wound healing rate of HOS cells (**Figure 3A**). Results of the Transwell invasion assay showed that overexpression of CKB increased invasion in U2-OS cells (**Figure 3B**, $P < 0.01$), while CKB silencing decreased invasion in HOS cells ($P < 0.01$). We used western blotting to detect the changes in the expression of MMP-7 and MMP-9 proteins. As illustrated in **Figure 3C**, the expression of MMP-7 and MMP-9 in U2-OS cells was increased upon overexpression of CKB ($P < 0.01$), while the opposite results were seen in HOS cells upon CKB silencing ($P < 0.01$).

CKB affects the activity of the p53 signaling pathway

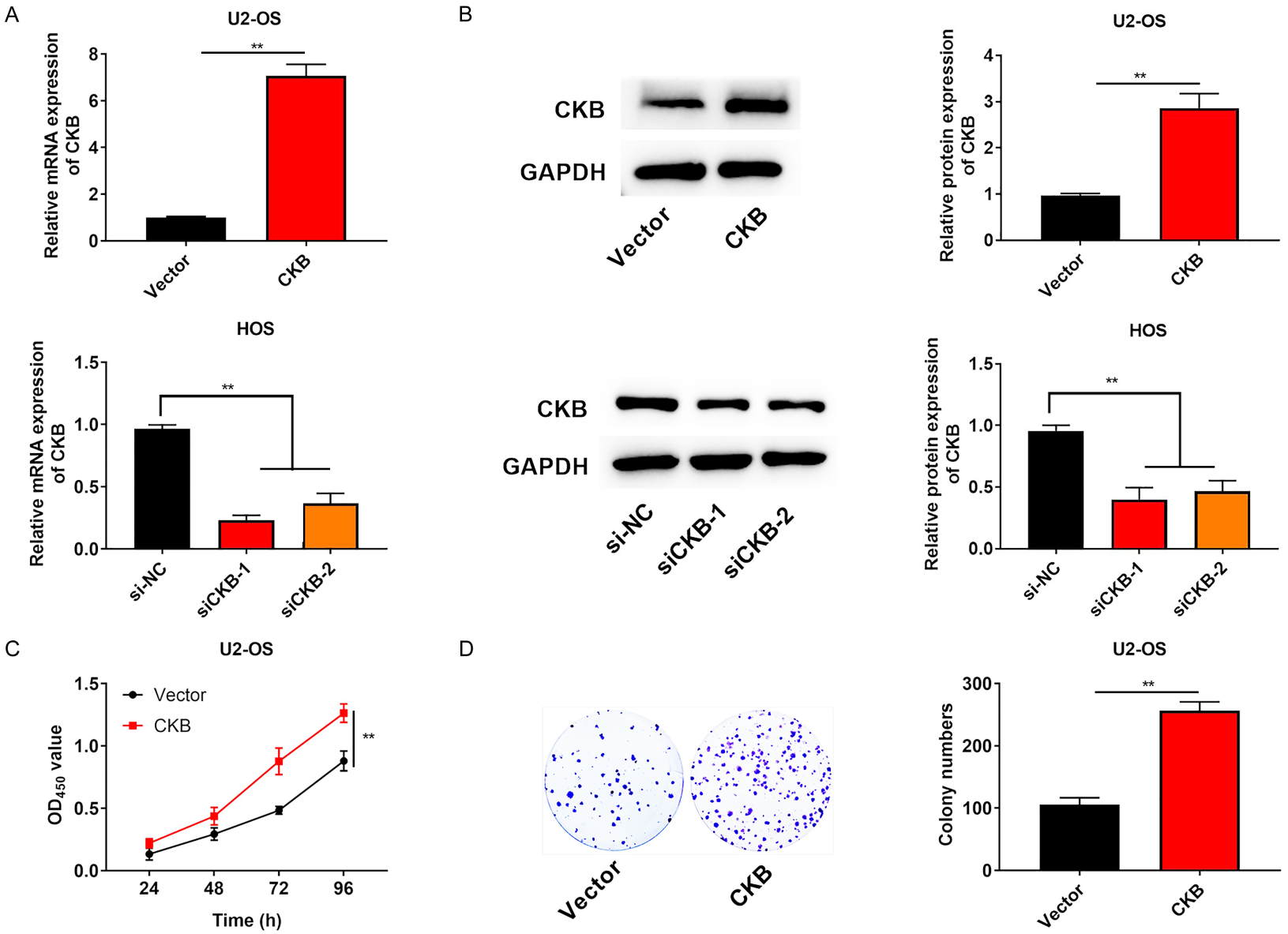
We used GSEA software to perform KEGG prediction and found that CKB could affect the p53 signaling pathway (**Figure 4A**). Next, the expression of p53 in OS cell lines (U2-OS, MG-63, SAOS-2, and HOS) and hFOB 1.19 cells was detected. As shown in **Figure 4B**, compared with that of hFOB 1.19 cells, the expression levels of p53 in OS cells were lower ($P < 0.01$). In U2-OS cells, the protein expression of p53, p21, and Bax was reduced upon overexpression of CKB, and the expression of Bcl-2 and MDM2 was increased upon overexpression of CKB (**Figure 4C**, $P < 0.01$). The silencing of CKB promoted the expression of p53, p21, and Bax, and inhibited the expression of Bcl-2 and MDM2 in HOS cells (**Figure 4C**, $P < 0.01$).

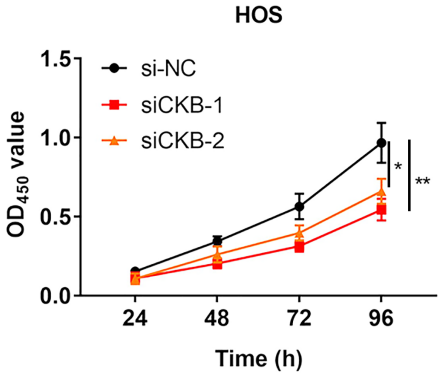
CKB affects the proliferation, apoptosis, and invasion of OS cells, which is involved in the activity of the p53 signaling pathway

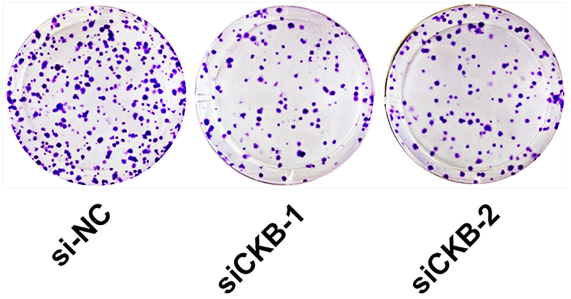
To further verify that CKB affected the development of OS through the p53 signaling pathway, we transfected p53 plasmids into U2-OS cells. As shown in **Figure 5A**, compared with the vector group, the expression of p53 protein in the p53 group was increased, and overexpression of p53 alleviated the effects of CKB overexpression. Cell proliferation and invasion were inhibited, and cell apoptosis was promoted upon overexpression of p53 (**Figure 5B-D**, $P < 0.01$). Overexpression of p53 reversed the effects of CKB overexpression. In addition, siRNA-p53 was transfected into HOS cells. As illustrated in **Figure 6A**, compared with the si-NC group, the expression of p53 protein was reduced in the si-p53 group, which indicated that the transfection was effective. Next, we transfected both siCKB-1 and si-p53 into HOS cells and found that p53 silencing alleviated the promotive effects of high levels of CKB (**Figure 6A**). Cell proliferation and invasion were promoted and cell apoptosis was inhibited by silencing of p53 (**Figure 6B-D**, $P < 0.01$). Silence of p53 reversed the effects of silencing CKB. The above experiments proved that CKB affected the development of OS cells through the p53 pathway.

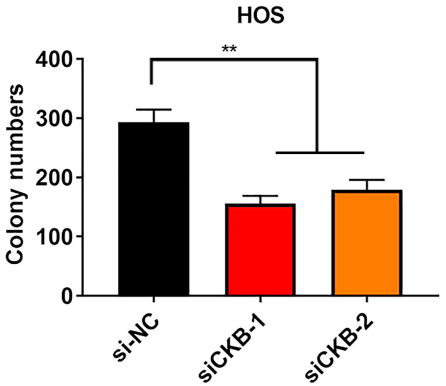
Discussion

OS is a primary tumor affecting bones that can occur at all ages, but has a high incidence in children and adolescents [22]. OS is characterized by high incidence and poor prognosis after surgery [2]. Therefore, the treatment of OS has been a hot topic in the medical and life science research. The current treatment methods for OS include complete surgical resection of the detected tumor site and multi-drug chemotherapies, however, these therapies lead to recurring incidents of OS and the poor prognosis of patients. Hence, in-depth research on OS is still needed.

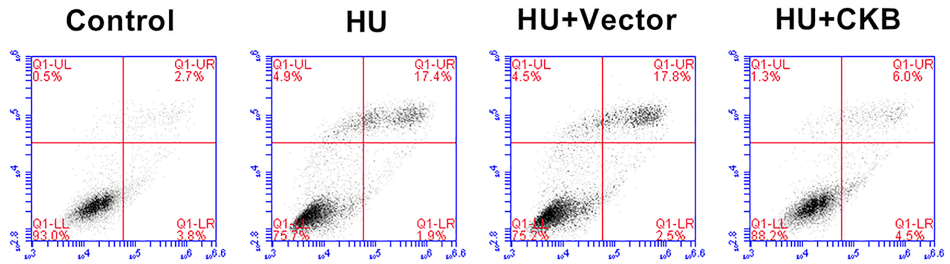

A
U2-OS
Relative mRNA expression of CKB
**
Vector
CKB
HOS
Relative mRNA expression of CKB
**
si-NC
siCKB-1
siCKB-2
B
CKB
GAPDH
Vector
CKB
CKB
GAPDH
si-NC
siCKB-1
siCKB-2
U2-OS
Relative protein expression of CKB
**
HOS
Relative protein expression of CKB
**
C
U2-OS
Vector
CKB
OD450 value
Time (h)
**
D
Vector
CKB
U2-OS
Colony numbers
**

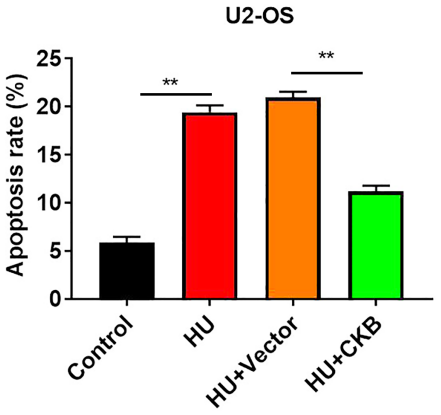

HOS
si-NC
siCKB-1
siCKB-2
OD_{450} value
Time (h)
24
48
72
96
*
**

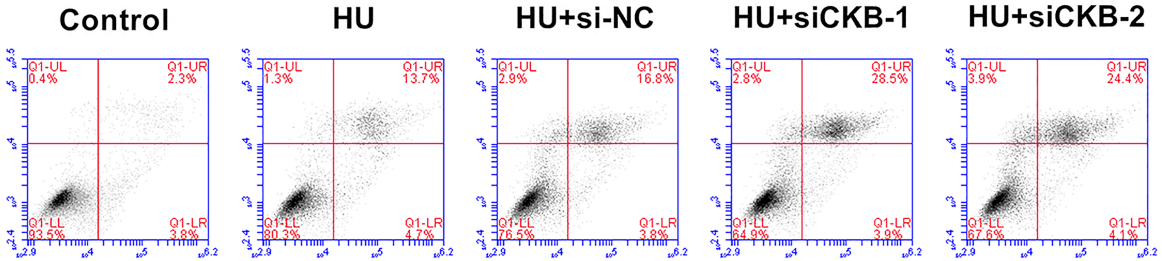

si-NC
siCKB-1
siCKB-2


HOS
Colony numbers
si-NC
siCKB-1
siCKB-2
**

E


Control
HU
HU+Vector
HU+CKB


U2-OS
Apoptosis rate (%)
Control
HU
HU+Vector
HU+CKB
**


Control
HU
HU+si-NC
HU+siCKB-1
HU+siCKB-2

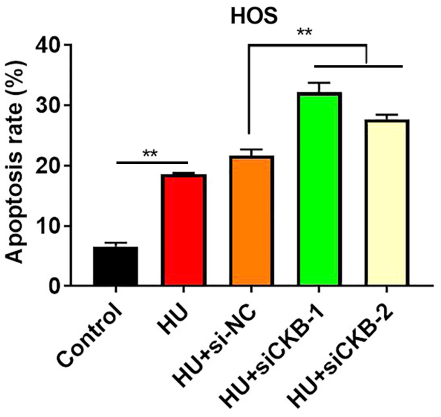

HOS
Apoptosis rate (%)
Control
HU
HU+si-NC
HU+siCKB-1
HU+siCKB-2
**

Figure 2. CKB affected the proliferation and apoptosis of OS cells. A. QRT-PCR was used to detect CKB mRNA expression in U2-OS and HOS cells; B. Western blotting was used to detect CKB expression in U2-OS and HOS cells; C and D. Cell proliferation in U2-OS and HOS cells was assessed using CCK-8 and colony formation assays; E. Cell apoptosis in U2-OS and HOS cells was measured using flow cytometry. *P < 0.05, **P < 0.01.

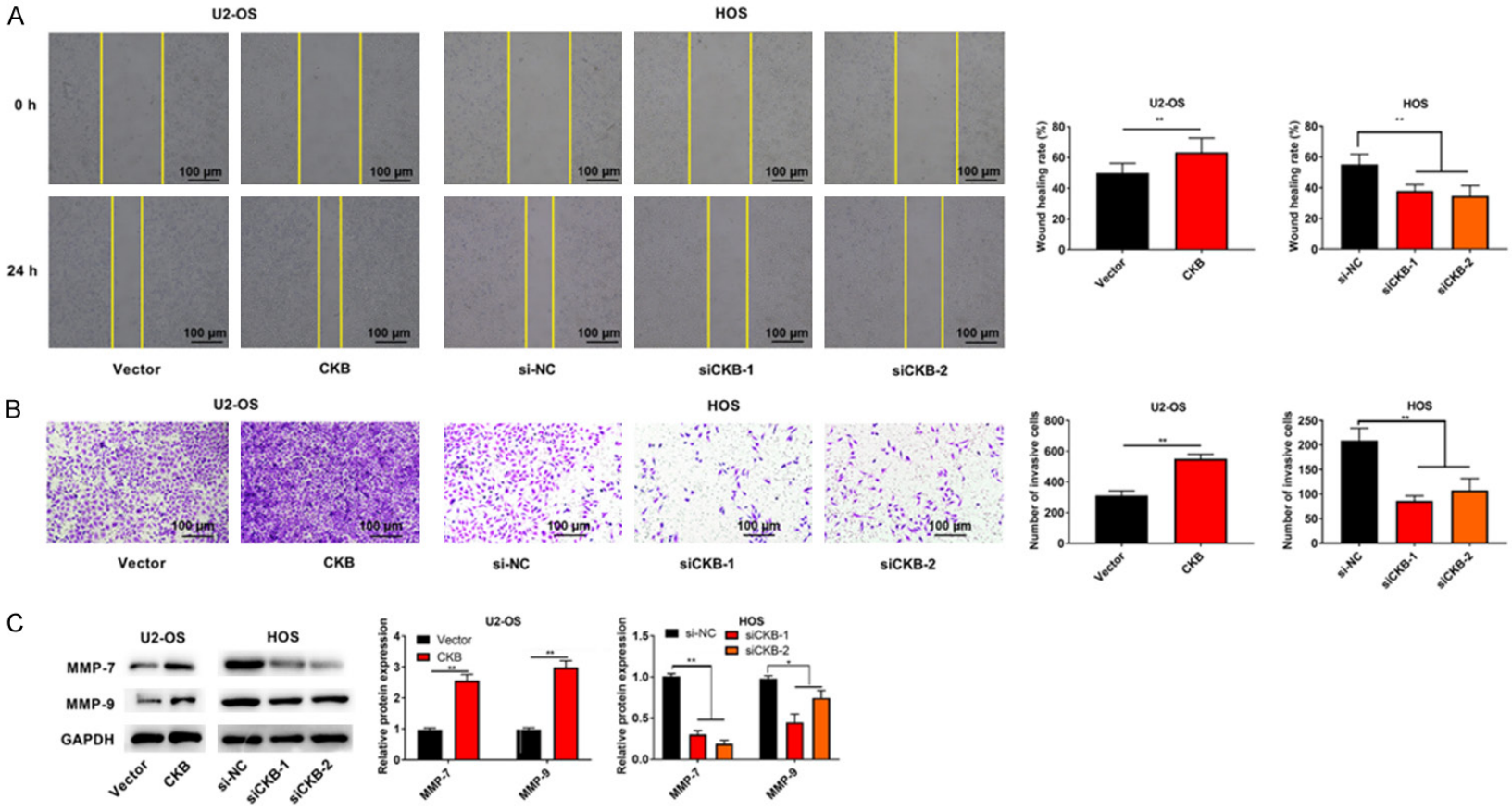


Figure 3. CKB affected the migration and invasion of OS cells. A. Cell migration in U2-OS and HOS cells was assessed using the cell scratch test; B. A Transwell experiment was used to detect cell invasion in U2-OS and HOS cells; C. Western blotting was used to detect the expression of MMP-7 and MMP-9 in U2-OS and HOS cells.*P < 0.05, **P < 0.01.

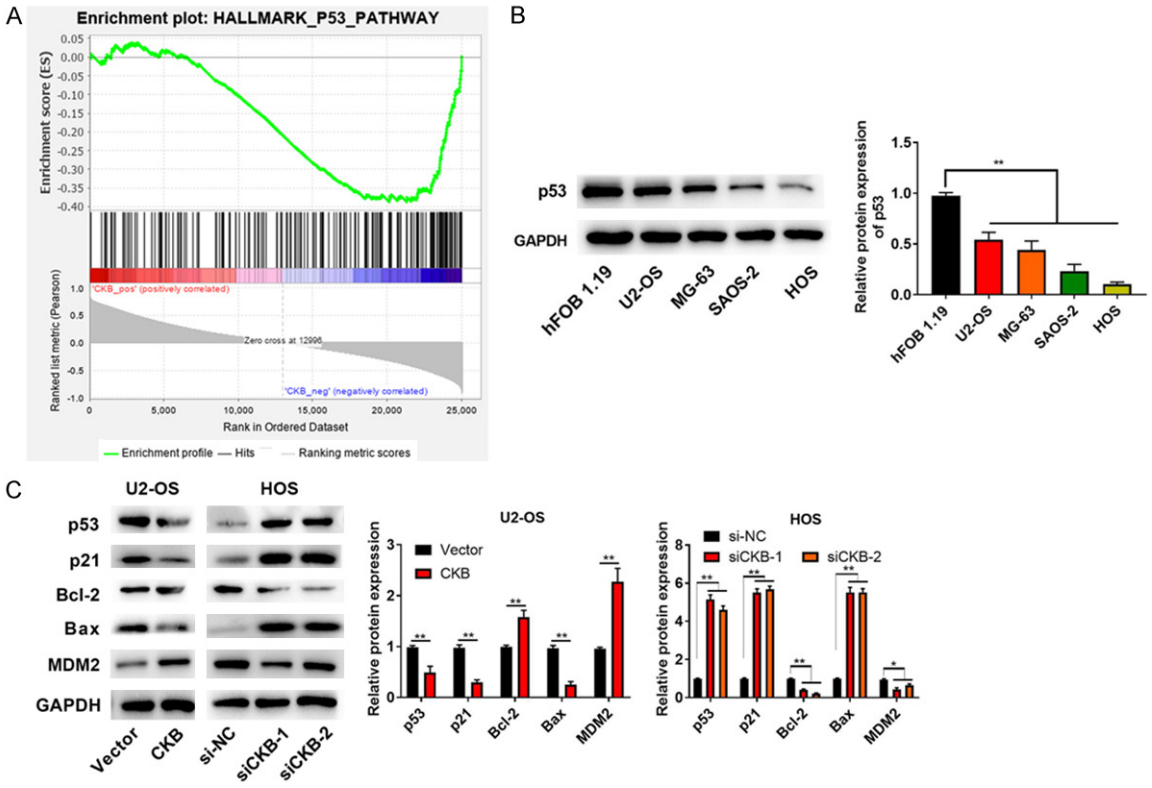
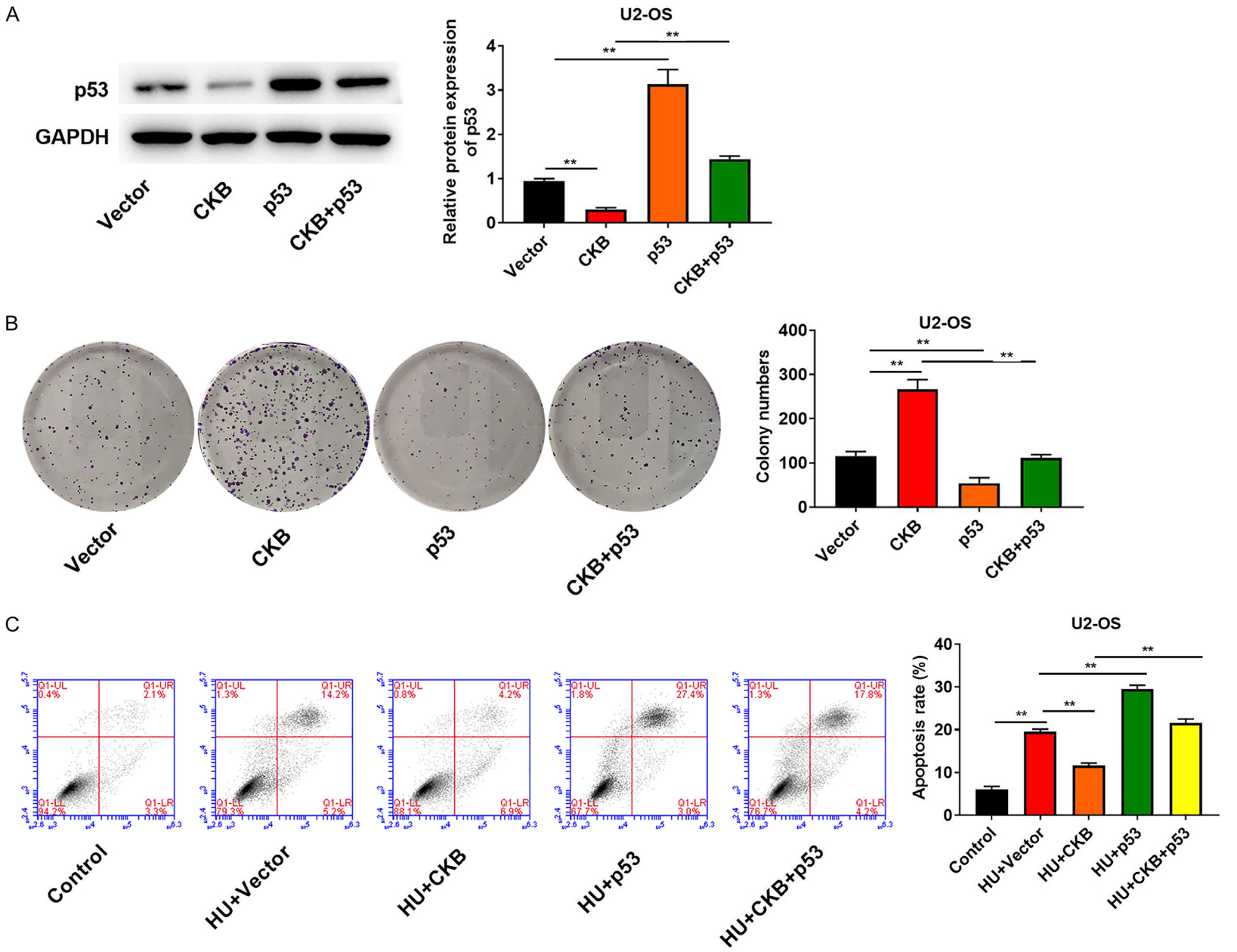


Figure 4. CKB inhibited the p53 signaling pathway. A. GSEA software analysis found that CKB could affect the p53 signaling pathway; B. p53 expression in the human osteoblast immortalized cell line (hFOB 1.19) and OS cell line (U2-OS, MG-63, SAOS-2, and HOS) was assessed using western blotting. C. Western blotting was performed to detect the levels of p53, p21, Bax, MDM2, and Bcl-2 in U2-OS and HOS cells. *P < 0.05, **P < 0.01.

In our study, the GEO data analysis showed elevated CKB expression in mesenchymal stromal cells of patients with OS. QRT-PCR and western blotting verified that CKB expression was indeed increased in OS tissues, suggesting that CKB might play an important role in the development of OS. Next, CKB was overexpressed and silenced to study the effects of CKB on OS. CCK-8 and colony formation experiments showed that silencing CKB expression weakened HOS cell proliferation ability. In addition, it was found that silencing CKB expression increased the apoptosis rate in HOS cells. Overexpression of CKB led to the opposite results. In previous reports, overexpression of CKB significantly increased the number of cells in the G2/M phase of Caco-2 colon cancer cells [23], which is consistent with our results. The above results proved that inhibiting CKB expression could attenuate OS cell proliferation and induce apoptosis.

Next, the migration and invasion ability of OS cells were assessed. CKB silencing inhibited HOS cell migration and invasion, while U2-OS cell migration and invasion were promoted by overexpression of CKB. The expression of MMP-7 and MMP-9, which are associated with metastasis, was also suppressed by CKB silencing. MMP-7 and MMP-9 are members of the MMP family, which are commonly expressed during tumor development and can participate in tumor cell migration and invasion [24]. The level of CKB in the MCF-7-MEK5 breast cancer cell line is upregulated, at which time the cell migration and invasion ability is activated [25]. The results of this study are consistent with ours, and both prove that inhibiting CKB expression can reduce tumor cell migration and invasion capabilities.

We also used GSEA software to analyze the signaling pathways related to CKB. We found that CKB activated some signaling pathways, such as E2F, MYC, and Wnt/β-catenin signaling pathways. In addition, CKB inhibited the p53 signaling pathway. We detected the changes in p53 pathway-related proteins using western blotting, and found that CKB silencing increased the expression of p53, p21, and Bax, and


A
p53
GAPDH
Vector
CKB
p53
CKB+p53
U2-OS
Relative protein expression of p53
B
Colony numbers
C
Control
HU+Vector
HU+CKB
HU+p53
HU+CKB+p53
Apoptosis rate (%)

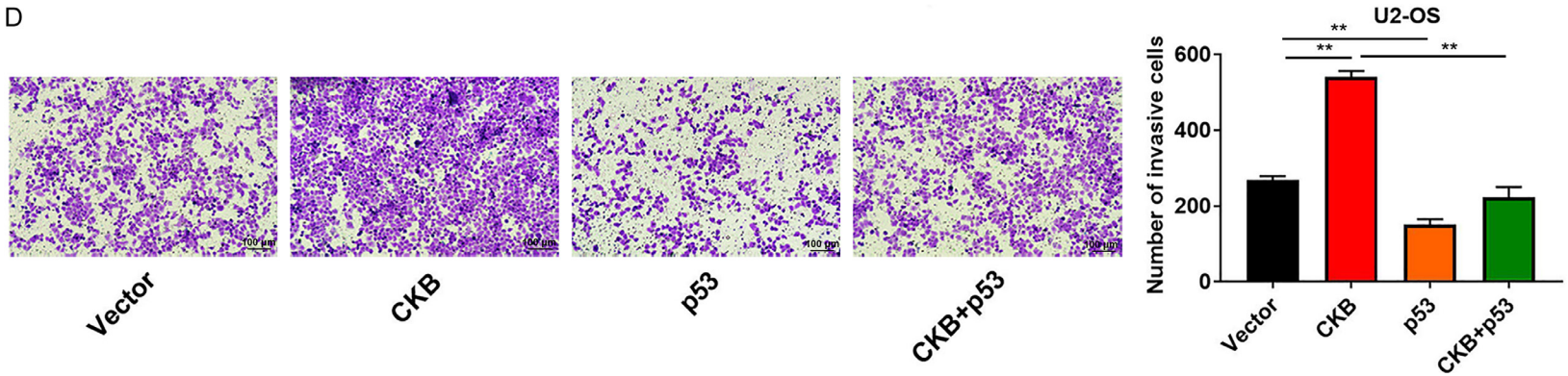
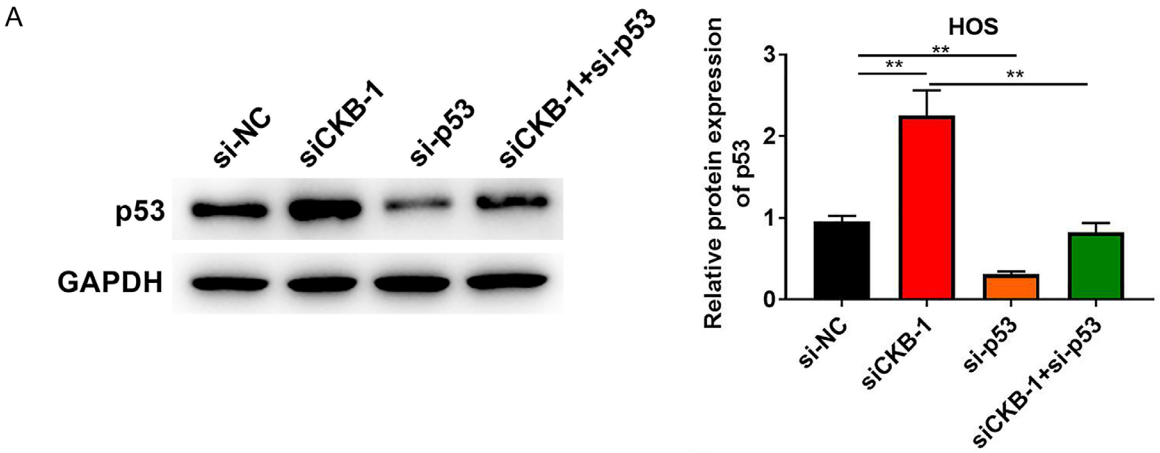


Figure 5. CKB affected U2-OS cell proliferation, invasion and apoptosis through the p53 signaling pathway. A. Western blotting was performed to measure the levels of p53 protein in U2-OS cells; B. U2-OS cell proliferation was assessed using colony formation assay; C. U2-OS cell apoptosis was measured using flow cytometry; D. A Transwell experiment was performed to assess U2-OS cell invasion. **P < 0.01.



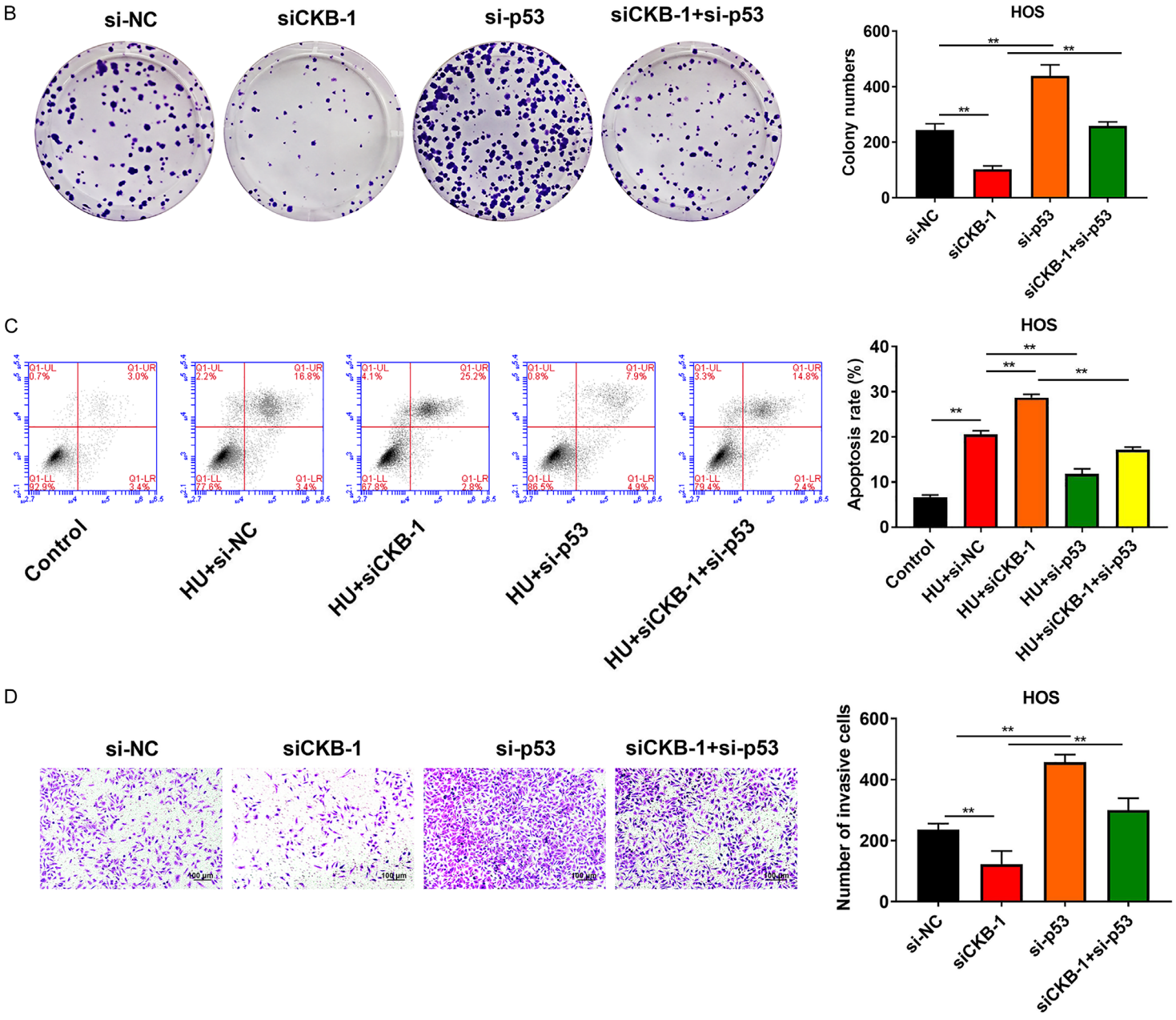

B
si-NC
siCKB-1
si-p53
siCKB-1+si-p53
HOS
Colony numbers
600
400
200
0
**
**
**
si-NC
siCKB-1
si-p53
siCKB-1+si-p53
C
Control
HU+si-NC
HU+siCKB-1
HU+si-p53
HU+siCKB-1+si-p53
HOS
Apoptosis rate (%)
40
30
20
10
0
**
**
**
**
Control
HU+si-NC
HU+siCKB-1
HU+si-p53
HU+siCKB-1+si-p53
D
si-NC
siCKB-1
si-p53
siCKB-1+si-p53
HOS
Number of invasive cells
600
400
200
0
**
**
si-NC
siCKB-1
si-p53
siCKB-1+si-p53

Figure 6. CKB affected HOS cell proliferation, invasion and apoptosis through the p53 signaling pathway. A. The expression of p53 protein in HOS cells was assessed using western blotting; B. HOS cell proliferation was assessed using colony formation assay; C. HOS cell apoptosis was measured using flow cytometry; D. A Transwell experiment was performed to detect HOS cell invasion. **P < 0.01.

decreased the expression of Bcl-2 and MDM2. Overexpression of CKB had the opposite effect. It has been reported that the increase in CKB expression in human lung tumor cells is accompanied by mutations to p53 alleles [26]. CKB knockdown inhibited ovarian cancer cell proliferation and induced apoptosis, which might be related to AKT inactivation [27]. Chen et al. reported that sesamin suppressed non-small cell lung cancer cell proliferation and induced apoptosis via the AKT/p53 pathway [28]. To further prove that CKB can affect the development of OS through the p53 signaling pathway, siRNA-CKB and siRNA-p53 were co-transfected into HOS cells. The number of colonies and invasive cells and apoptosis rate all changed, and the inhibitory effect of CKB silencing on HOS cells was alleviated by siRNA-p53. In addition, CKB and p53 plasmids were co-transfected into U2-OS cells. The results showed that the facilitation effect of CKB on U2-OS cell proliferation and invasion were alleviated by p53. This further proved that CKB could affect the development of OS by regulating the activity of the p53 signaling pathway.

Conclusions

In summary, CKB was shown to affect OS development by regulating the activity of the p53 signaling pathway. This study proposes the role of CKB in human OS for the first time and clarifies the specific mechanisms underlying OS development. Therefore, this research has important clinical research significance and can provide a more detailed theoretical basis and experimental basis for the control of tumor development in the future.

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The protocol of this research has been approved by the Ethics Committee of Tianjin Integrative Medicine Hospital (Tianjin Nankai Hospital). All patients have signed written informed consent.

Disclosure of conflict of interest

None.

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