Lentivirus-mediated MDA7/IL24 expression inhibits the proliferation of hepatocellular carcinoma cells

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Abstract. MDA7/IL24 is a member of the IL-10 gene family that functions as a cytokine. Notably, supra-physiological endogenous MDA7 levels have been indicated to suppress tumor growth and induce apoptosis in different cancer types. In the present study, MDA7 roles were investigated during the proliferation of hepatocellular carcinoma (HCC) cells and the molecular mechanisms underlying this process. A lentiviral vector expressing MDA7/IL24 (LV-MDA7/IL24) was constructed and used to infect HCC SMMC-7721 cells. The expression levels of *MDA7/IL24* in these cells were determined using RT-qPCR and western blot analysis. The effects of LV-MDA7/IL24 on cell proliferation were analyzed using MTT and colony formation assays. Furthermore, the influence of LV-MDA7/IL24 on cell apoptosis and cell cycle distribution were detected using flow cytometry. The underlying molecular mechanisms were investigated using microarray and western blot analysis. The expression of *MDA7/IL24* was confirmed to be significantly increased in the cells infected with LV-MDA7/IL24 compared with that the negative-control infected group. Lentivirus-mediated *MDA7/IL24* expression was found to inhibit HCC cell proliferation and colony formation, and it also induced cell arrest and apoptosis. Microarray analysis and western blotting results indicated that multiple cancer-associated pathways and oncogenes are regulated by MDA7/IL24, including cell cycle regulatory and apoptosis activation pathway. In conclusion, it was determined that MDA7/IL24 inhibits the proliferation and reduces the tumorigenicity of HCC cells by regulating cell cycle progression and inducing apoptosis, indicating that it may be used as a potential prognostic and therapeutic target in HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the most common malignancy of the liver and the third leading cause of cancer-related death worldwide. Despite the development of various therapies, the outcome for HCC patients remains poor. Generally, HCC patients have a 1-year survival rate of <50% and a 5-year survival rate of 10% (1). Annually, ~0.11 million people die from liver cancer in China, and the major reason for frequent HCC relapses are intrahepatic and distant metastases that develop after the curative surgical resection or transplantation (2). Moreover, HCC is insensitive to chemotherapy and radiotherapy (3). Therefore, novel HCC treatments such as gene therapy and molecular targeted therapy should be investigated (4). As a multitarget anticancer drug, sorafenib has been approved for the treatment of HCC, but showed a very low success rate (5). Gene therapy represents an alternative form of cancer treatment and was shown to have high efficiency, specificity, and few serious side effects (4).

Melanoma differentiation-associated gene7/interleukin-24 (MDA7/IL24), a member of the IL10 cytokine family, was originally identified as a gene associated with the terminal differentiation and irreversible growth suppression of meta-static human melanoma cells (6). MDA7/IL24 is considered to be secreted by the immune system and melanocytes alone (7,8). Several reports demonstrated the loss of *MDA7/IL24* expression during the progression of melanoma, and a significant inverse correlation between the loss of this gene and tumor invasion, suggesting that MDA7/IL24 may have anticancer effects (6,7,9,10). Additionally, our previous studies demonstrated that MDA7/IL24 has multiple anticancer functions, selectively inducing cancer cell apoptosis, but also showing immunomodulatory and antiangiogenic properties and strong antitumor bystander effects, which makes this molecule an ideal candidate for cancer gene therapy (9-13).

We constructed MDA7/IL24-expressing lentiviral particles, and evaluated the effects of lentivirus-mediated MDA7/IL24 expression on HCC cell proliferation and colony-forming ability. Moreover, we explored the mechanisms underlying MDA7/IL24-mediated HCC regression (14).

Materials and methods

Cell lines and culture conditions. HCC cell line SMMC-7721 was obtained from Cell Bank of Chinese Academy of Sciences

(Shanghai, China), and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml of penicillin-streptomycin. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. In addition, the cell line is not contaminated or mis-identified according to the Database of Cross-Contaminated or Misidentified Cell Lines.

Recombinant lentiviral particle construction and infection. We constructed *MDA7/IL24* gene expression plasmid, while an empty plasmid was used as a negative control. Following this, *MDA7/IL24*-expressing plasmid or the negative control plasmid, together with pHelper 1.0 and pHelper 2.0 (pVSVG-I and pCMVΔR 8.92 plasmids, respectively), were added to 293T cells with Lipofectamine 2000 (Invitrogen, Shanghai, China), according to the manufacturer's instructions. After 48 h of transfection, supernatants containing viral particles were collected and centrifuged (1,006 g, 20 min) to get rid of cell debris, and filtered through 0.45-μm polyvinylidene fluoride (PVDF) membranes. HCC cells were infected with *MDA7/IL24*-expressing lentiviral particles or the controls at the multiplicity of infection (MOI) of 20. The infected cells expressing green fluorescent protein (GFP) were observed under a fluorescence microscope (Micro Publisher 3.3RTV; Olympus, Tokyo, Japan). The cells were collected and total RNA was extracted to determine the efficiency of knockdown.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted and purified from SMMC-7721-infected cells, using Trizol reagent (Invitrogen) following the manufacturer's instructions. RT was performed to generate cDNA molecules, using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and oligo(dT) primers (Sangon, Shanghai, China), following the manufacturers' instructions. The expression of *MDA7/IL24* was determined by quantitative real-time (qRT-) PCR, using a PCR assay kit (TransGen Biotech, Beijing, China). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA was amplified as an internal reference. MDA7/IL24 primer set: Forward, 5'-TTGCCTGGGTTTTACCCTGC-3' and reverse, 5'-AAGGCTTCCCACAGTTTCTGG-3'; GAPDH forward, 5'-TGACTTCAACAGCGACACCCA-3' and reverse, 5'-CAC CCTGTTGCTGTAGCCAAA-3'. PCR conditions included initial denaturation (95°C for 10 sec) and then 40 cycles of amplification (95°C for 15 sec and 55°C for 15 sec). *MDA7/IL24* relative expression was normalized to *GAPDH* levels by the $2^{-\Delta\Delta Ct}$ method (15).

MTT assay. To investigate the effects of *MDA7/IL24* over-expression on cell viability, MTT assay was performed three times. SMMC-772 cells in the logarithmic growth phase were cultured for 24 h in 96-well plates (1×10^5 cells per well). After the infection, cells were incubated for additional 72 h. Mitochondrial function was evaluated by MTT colorimetric assay. Briefly, the medium was removed and a fresh medium containing 0.5 mg/ml MTT was added to each well. The cells were incubated at 37°C for 4 h. Following this, the supernatants were removed, 50 μl dimethylsulfoxide (DMSO) was added to each well, and samples were incubated for 30 min at 37°C with gentle shaking. Finally, absorbance was determined using a microplate reader at 490 nm. Cell viability was calculated as the ratio of the absorbance determined in the samples infected with the *MDA7/IL24* overexpression plasmid to that of the control group (untreated cells).

Colony formation assay. Infected and untreated SMMC-7721 cells were plated in six-well plates (200 cells/well) and cultured in a 5% CO_2 incubator at 37°C for 14 days. The cells were washed twice with PBS and fixed in 4% paraformalde-hyde for 30 min. Cell colonies were stained with Giemsa dye (Chemicon, Temecula, CA, USA) for 20 min, and washed with double distilled water several times. Colony numbers were counted under a fluorescence microscope.

Cell cycle. Cells were cultured in 12-cell plates. After 5 days, the cells were collected and fixed with cold 70% ethanol overnight at -20°C, and then washed with cold PBS for one time. The fixed cells were treated with RNase and stained with propidium iodide (Sigma, St. Louis, MO, USA). The stained cells were analyzed by flow cytometer and ModFit LT software (Verity Software House, Topsham, ME, USA).

Cell apoptosis. Cell apoptosis was performed using Annexin V PE and 7-AAD apoptosis detection kit (BD Bioscience, San Diego, CA, USA) according to the manufacturer's instructions. Cells were collected after cultured 5 days, washed and resuspended with 1xbinding buffer. Then 5 μl Annexin V was added into 200 μl of the above cell suspension and incubated at room temperature in the dark for 15 min. After incubation, 5 μl 7-AAD was added the cell apoptosis was detected using the flow cytometer.

Microarray processing and analysis. Total RNA isolated from SMMC-7721 cells infected with either lentiviral vector expressing (LV-)MDA7/IL24 (n=3) or negative control lenti-virus (n=3) was subjected to microarray analysis, to determine the global transcriptomic profile of each cell group, using Affymetrix human GeneChip according to the manufacturer's instructions. Microarray hybridization, washing, and staining were performed using the GeneChip Hybridization Wash and Stain kit (Affymetrix, Santa Clara, CA, USA). Arrays were then scanned using the GeneChip Scanner 3,000 to obtain raw data (Affymetrix). Significant differences in the expression of the analyzed genes between SMMC-7721 cells infected with either LV-MDA7/IL24 or negative control lentivirus were obtained based on P<0.05.

Western blot analysis. Seventy-two h after the infection of SMMC-7721 cells with either LV-MDA7/IL24 or negative control lentivirus, the cells were collected and washed with PBS twice, and the lysis buffer was added to extract the cellular proteins. Afterwards, the lysates were centrifuged at 14,000 rpm at 4°C for 10 min and the supernatants were collected. BCA method was applied to determine the protein concentration. Twenty micrograms of protein sample obtained from the infected cells was separated by 10% SDS-PAGE and then transferred to PVDF membrane. PVDF membranes were blocked at 4°C overnight with 5% bovine serum albumin and incubated with monoclonal antibodies against
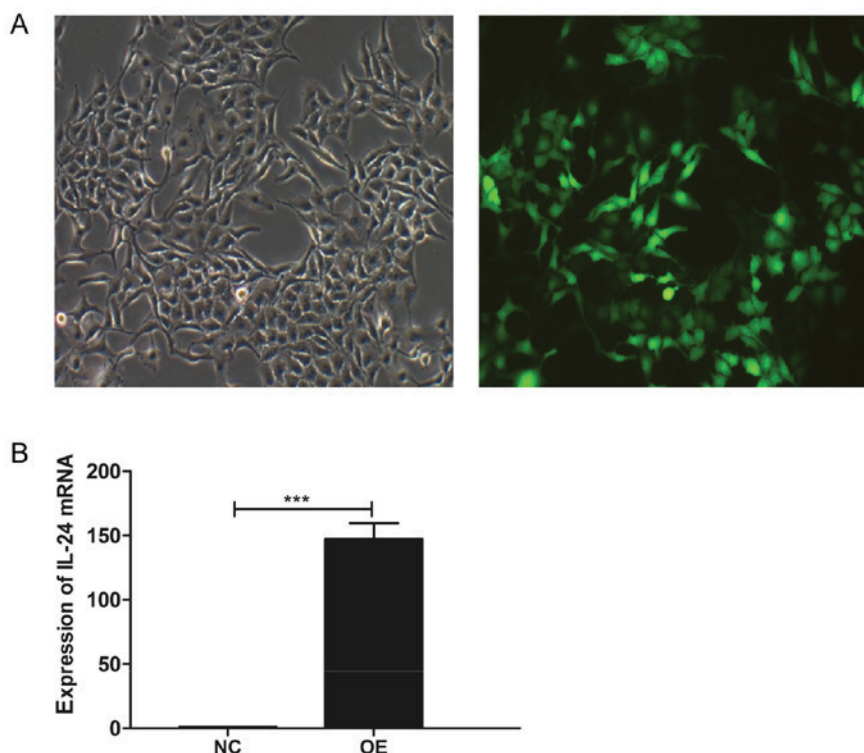
Figure 1. SMMC-7721 cells infected with LV-MDA7/IL24 or negative-control lentiviral particles. (A) Bright-field and fluorescent photomicrographs after the culturing of the infected cells for 3 days. Magnification, x100. (B) *MDA7/IL24* mRNA expression levels in LV-MDA7/IL24-infected (OE) and negative control (NC) cells. Gene expression levels were normalized to that of the *GAPDH*. Data are presented as mean ± SD obtained in three independent experiments (***P<0.001). LV-MDA7/IL24, lentiviral vector expressing MDA7/IL24; *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase.
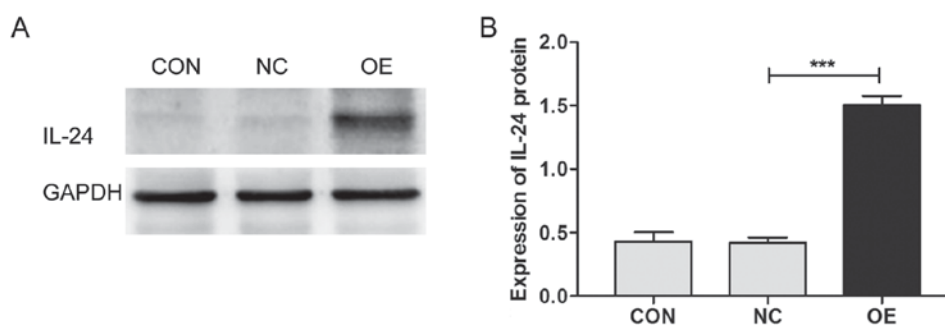

Figure 2. *MDA7/IL24* protein expression in LV-MDA7/IL24-infected SMMC-7721 cells. (A) Western blot analysis indicated the protein expression of *MDA7/IL24* in LV-MDA7/IL24-infected SMMC-7721 cells. (B) The densitometric and statistical analysis of western blot results in LV-MDA7/IL24-infected (OE), negative control (NC) and untreated cells (CON). Data are presented as mean ± SD obtained in three independent experiments (***P<0.001). LV-MDA7/IL24, lentiviral vector expressing MDA7/IL24.

B cell lymphoma protein-2 (BCL2; 1:500; ab692; Abcam, Cambridge, UK), Cyclin E (1:1,000; cat. no. 4132), p-ERK1/2 (1:800; cat. no. 4370), p-AKT (1:2,000; cat. no. 4060) and caspase-3 (1:1,000; cat. no. 9662; all CST Biological Reagents Co., Ltd., Shanghai, China). Thereafter, the horseradish peroxidase-conjugated secondary antibody we added for each corresponding primary antibody. The obtained blots were analyzed using enhanced chemiluminescence. GAPDH was detected on the same membrane as a loading control.

Statistical analysis. Student's t-test was performed for data analysis. Statistical analysis was performed using the SPSS version 22.0 software (SPSS, Chicago, IL, USA). All data were presented as mean ± standard deviation (SD)

of the results obtained in three independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

LV-MDA7/IL24 infection induces MDA7/IL24 overexpression in SMMC-7721 cells. As presented in Fig. 1A, SMMC-7721 cells were shown to be GFP-positive following the infection with the lentiviral particles, indicating a high efficiency of the infection. Further analysis demonstrated a significant upregulation of *MDA7/IL24* expression in these cells (P<0.001), compared with that in the cells infected with the negative control lentiviruses (Figs. 1B and 2).
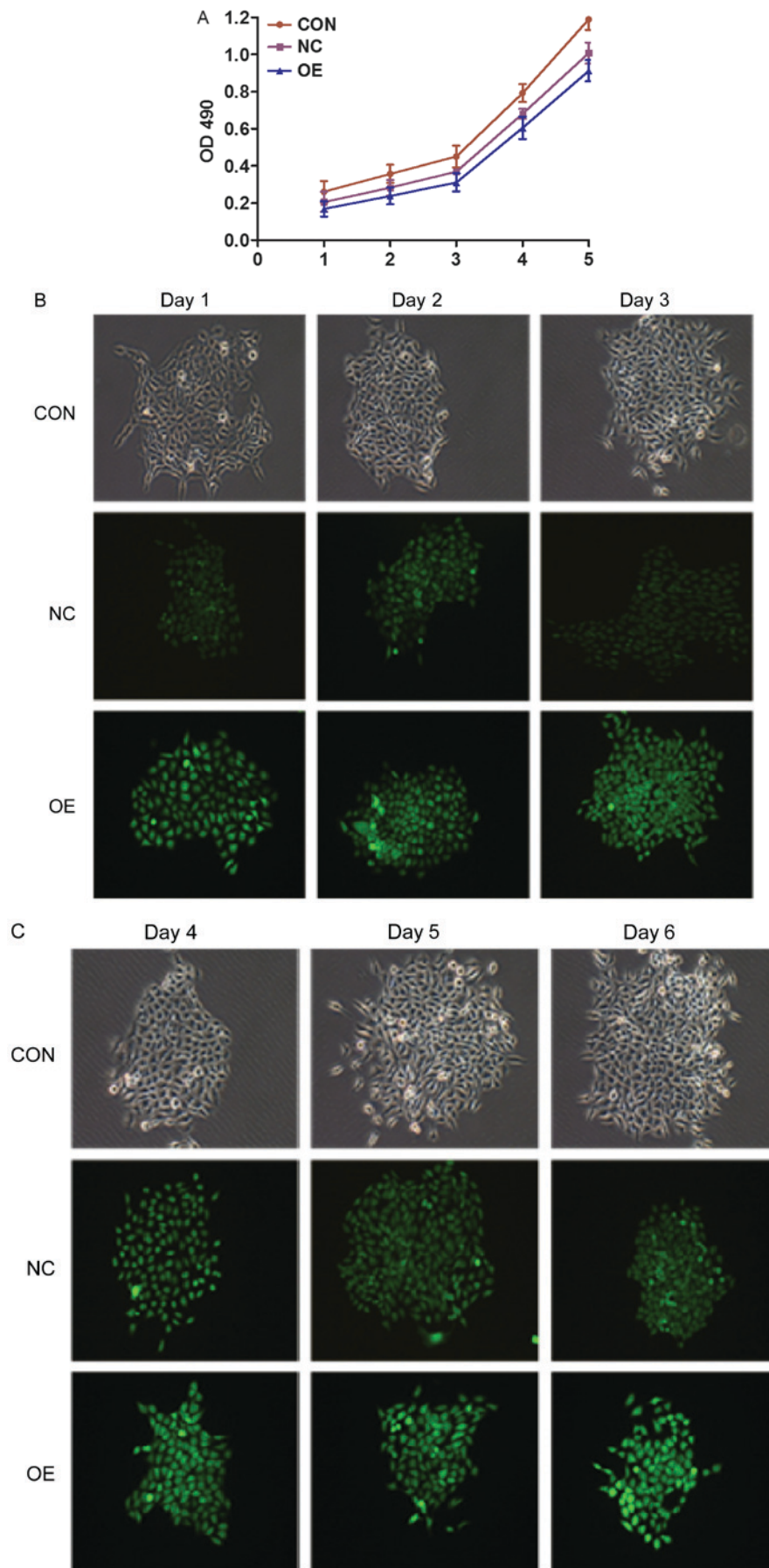
Figure 3. *MDA7/IL24* overexpression suppresses SMMC-7721 cell proliferation. (A) Cell proliferation of LV-MDA7/IL24-infected cell, negative control-infected cells, and untreated cells. (B and C) The photomicrographs of SMMC-7721 cells at different time points, including the fluorescent images of experimental group cells and the bright-field images of the control group cells. Magnification, x100. OE, cells infected with LV-MDA7/IL24; NC, cells infected with the negative control particles; CON, untreated cells.
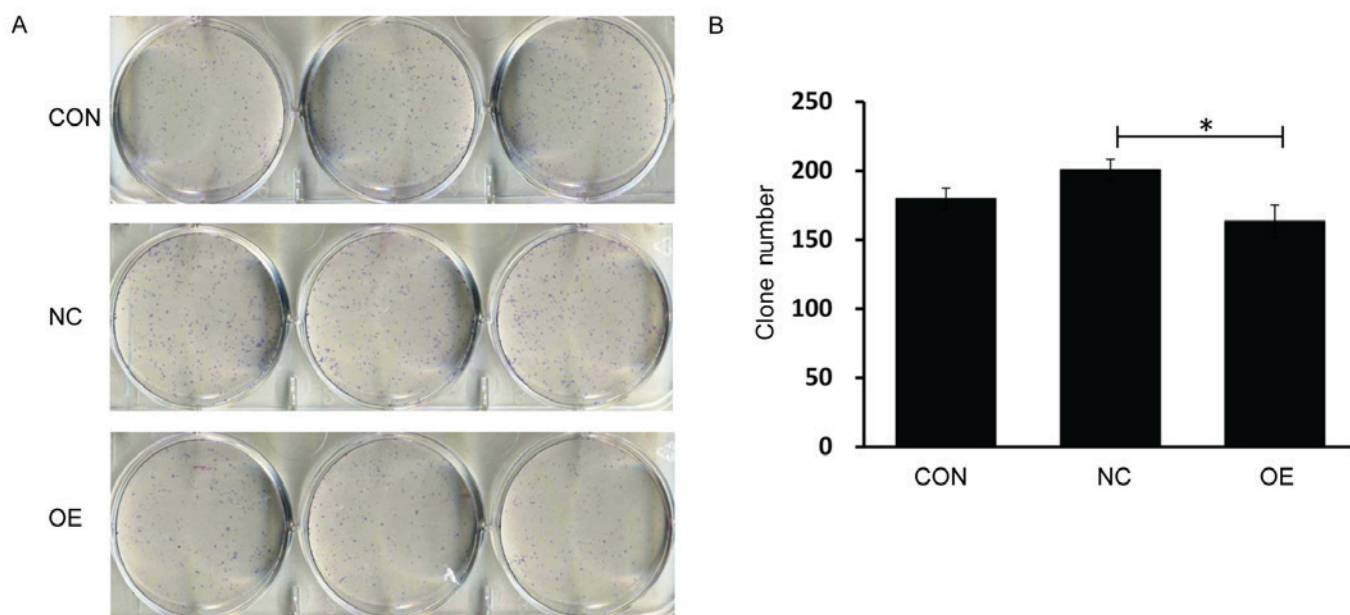
Figure 4. *MDA7/IL24* overexpression reduces SMMC-7721 cell colony formation. (A) Colony formation assay results and crystal violet staining of SMMC-7721 colonies. (B) Quantification of cells in each colony. OE, cells infected with LV-MDA7/IL24; NC, cells infected with the negative control particles; CON, untreated cells. *P<0.05 as indicated.
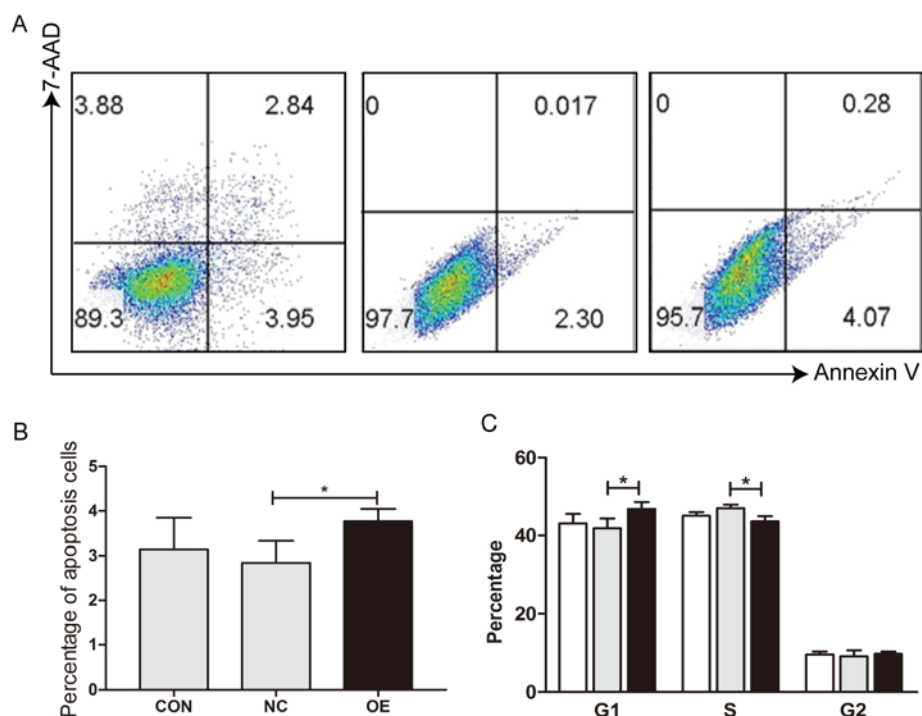

Figure 5. MDA7/IL24 overexpression induces SMMC-7721 cell apoptosis and cell cycle arrest. (A and B) The percentage of apoptosis cells in three different groups (*P<0.05). (C) The distribution of three groups of cells in the cell cycle (*P<0.05). OE, cells infected with LV-MDA7/IL24; NC, cells infected with the negative control particles; CON, untreated cells.

MDA7/IL24 overexpression inhibits SMMC-7721 cell prolife-ration and colony-forming ability. LV-MDA7/IL24-infected SMMC-7721 cells were shown to have a decreased growth rate, in comparison with the untreated and negative control-treated cells (P<0.05). At day 5, we determined that the optical density at 490 nm (OD490) of LV-MDA7/IL24-infected cells was 0.921±0.013, while those of the negative control-infected and untreated cells were 0.988±0.007 and 1.094±0.007, respec-tively (Fig. 3). Colony-forming ability of SMMC-7721 cells was analyzed by crystal violet staining, and the number of cells per colony significantly decreased after LV-MDA7/IL24 infection (P<0.05). Furthermore, we determined the number of colonies, and this number was found to decrease to 163±12 in samples overexpressing *MDA7/IL24*, compared with those
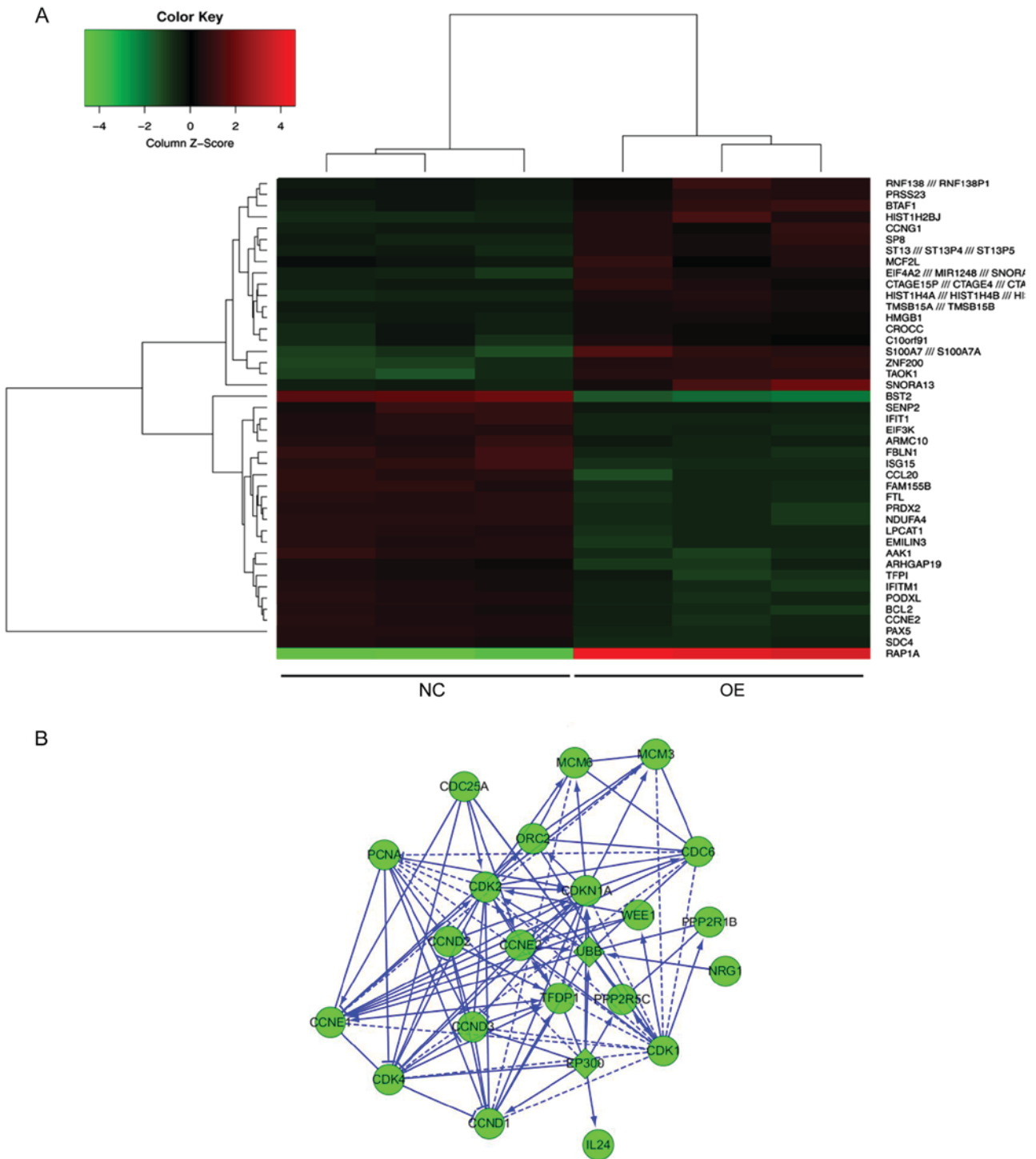
Figure 6. *MDA7/IL24* overexpression leads to changes in gene expression profile of SMMC-7721 cells. (A) Gene expression profiling results. Heatmap represents 43 genes shown to be differentially expressed between SMMC-7721 cells infected with negative-control (NC) lentivirus or LV-MDA7/IL24 (OE). Genes and samples are listed in rows and columns, respectively. Color scale: red represents gene expression upregulation; green, downregulation of gene expression. (B) Schematic representation of a network including MDA7/IL24 and genes involved in cell cycle regulation and apoptosis. Solid arrows, confirmed regulatory relationships; dotted lines, predicted regulatory relationships; T bars, inhibitory relationships.

in the untreated and negative control-treated cells (180±8 and 201±8, respectively) (Fig. 4).

MDA7/IL24 overexpression induces cell arrest and apoptosis. To demonstrate the reasons why MDA7/IL24 overexpression results in reduced cell viability, apoptosis and cell cycle of MDA7/IL24 positive and negative HCC cells were examined by flow cytometry. The results showed that MDA7/IL24 overexpression induced cell apoptosis (Fig. 5A and B) and elevated the percentage of G1 phage cells in SMMC-7721 cells (Fig. 5C).

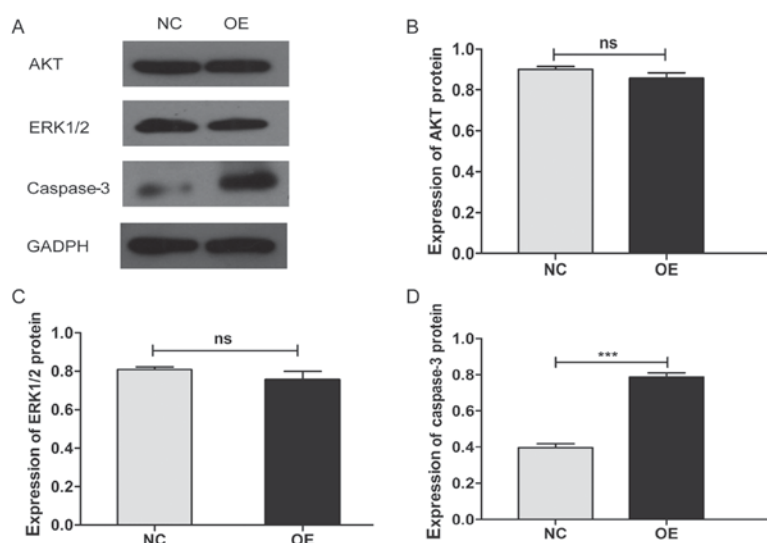*MDA7/IL24 overexpression affects multiple cancer development pathways.* To explore the molecular mechanisms

Figure 7. (A) Western blot analysis showing the expression levels of (B) AKT, (C) caspase-3 and (D) ERK1/2 in NC and OE cells. ***P<0.001. ns, not significant; OE, cells infected with LV-MDA7/IL24; NC, cells infected with the negativecontrol particles; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
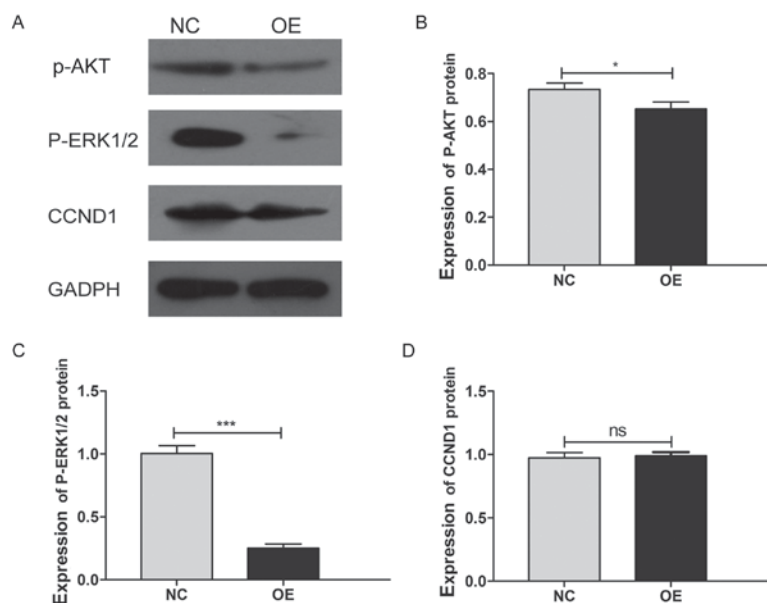

Figure 8. (A) Western blot analysis showing the expression levels of (B) p-AKT, (C) p-ERK1/2, (D) CCND1 in NC and OE cells. GAPDH was used as the loading control. *P<0.05; ***P<0.001. ns, not significant. OE, cells infected with LV-MDA7/IL24; NC, cells infected with the negative control particles; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

underlying the anticancer effects of MDA7/IL24, we performed microarray analysis and gene expression profiling in cells infected with either LV-MDA7/IL24 or the negative control. A significant difference in the expression levels between these cells was observed for 43 genes, 20 with upregulated and 23 with downregulated expression (Fig. 6A). KEGG pathway analysis demonstrated that these genes were significantly enriched in three pathways, including cell cycle regulation, DNA synthesis and transcriptional and apoptosis (Fig. 6B). Additionally, we verified the differential expression of several key molecules involved in these two pathways by western blotting, which showed that the upregulation of *MDA7/IL24* induces the expression of caspase-3 and downregulates the expression of p-AKT, p-ERK1/2, CCNE2, and BCL2 (Figs. 7-10).

Discussion

Gene therapy has become a focus of current investigations aimed at improving HCC treatment (16). MDA7/IL24 is a member of the IL10 cytokine family, and was shown to induce apoptosis in different cancers specifically, including HCC (17), lung cancer (18), melanoma (19), breast cancer (20), pancreatic cancer (21), cervical cancer (22), and prostate cancer (23), but it does not affect normal cells (24). Therefore, this gene may represent an ideal gene therapy target (25). Additionally, MDA7/IL24 can induce the activation of immune system response aimed against cancer cells, and effectively inhibit neoplastic angiogenesis. It was demonstrated that the overexpression of this molecule
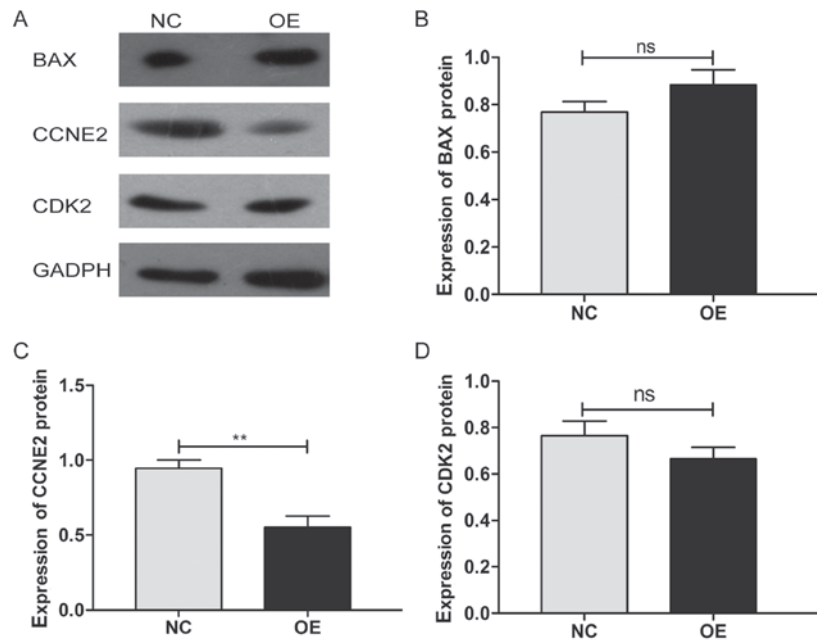
Figure 9. (A) Western blot analysis showing the expression levels of (B) Bax, (C) CCNE2 and (D) CDK2 in NC and OE cells. GAPDH was used as the loading control. **P<0.01. ns, not significant; OE, cells infected with LV-MDA7/IL24; NC, cells infected with the negative control particles; GAPDH, glyceralde-hyde-3-phosphate dehydrogenase.

Figure 10. (A) Western blot analysis showing the expression levels of and (B) C-PARP, (C) BCL2 and (D) PCNA in NC and OE cells. GAPDH was used as the loading control. ***P<0.001. ns, not significant; OE, cells infected with LV-MDA7/IL24; NC, cells infected with the negative control particles; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
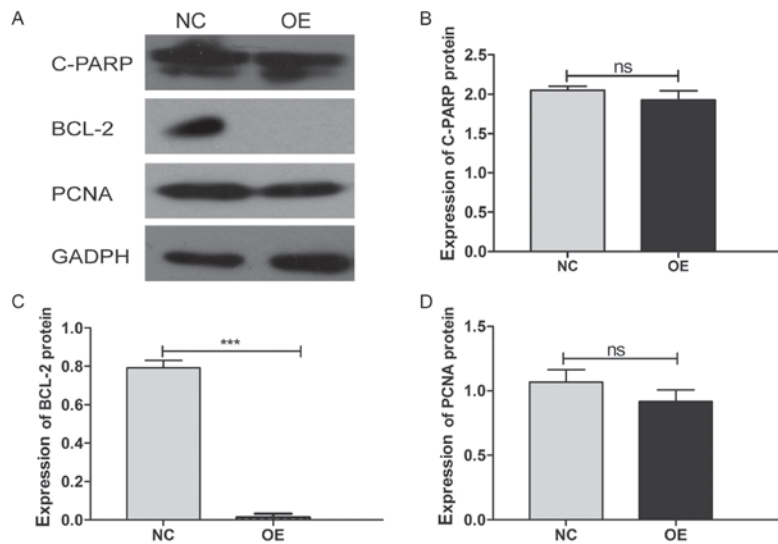
and chemotherapy have synergistic effects (26). However, *MDA7/IL24* overexpression mediated by lentiviruses has not been investigated previously.

Here, we constructed a *LV-MDA7/IL24*, and confirmed that the expression levels of this gene increase in SMMC-7721 cells infected with lentiviral particles carrying this vector. This demonstrated that the recombinant lentiviruses effectively promote the expression of *MDA7/IL24* in HCC cells. MTT and colony formation assays demonstrated that lentivirus-mediated *MDA7/IL24* expression markedly inhibits HCC cell proliferation. The number of cells and cell growth rate of LV-MDA7/IL24-infected cells were shown to be significantly

decreased. In accordance with the results obtained in a previous study (27), we demonstrated the anticancer effects of MDA7/IL24.

However, the molecular mechanisms underlying the effects of MDA7/IL24 on HCC cells remain unclear. We performed gene expression profiling of SMMC-7721 cells infected with LV-MDA7/IL24, demonstrating that many genes show significantly different expression between *MDA7/IL24*-overexpressing cells and the control cells. We further performed functional pathway analysis, and several pathways involving the differentially expressed genes were found to be involved in cancer development (14), for example,

G1/S checkpoint and G2/M DNA damage signaling pathway. These pathways are crucial for cell cycle regulation, DNA synthesis, and transcription. Previously, it was reported that MDA7/IL24 induces IL20/IL22 receptor-independent apoptosis by modulating multiple apoptotic signaling pathways such as mitochondrial pathway, MAPK, PKR, GADD pathways, and others (28-30), and is involved in the accumulation of BAX and BCL2 (31). The results of our western blot analysis showed that MDA7/IL24 induces the expression of caspase-3 and inhibits the expression of p-AKT, p-ERK1/2, CCNE2, and BCL2.

CCNE2 and p-ERK1/2 are molecules involved in cell cycle regulation, which can induce tumor progression by regulating cell cycle transition. Cyclin E2 is a member of cyclin E family, which forms cyclin E-CDK2 complex with CDK2 (6). This complex promotes cell cycle progression by regulating G1/S phase transition, and the dysregulation of cyclin E2-CDK2 activity was shown to be involved in tumor development (32). Moreover, the overexpression of cyclin E2 was shown to be associated with poor survival of breast cancer patients (33). P-ERK1/2 is the activated form of ERK1/2, which plays an important role in cell proliferation by regulating cell cycle progression (34). P-ERK1/2 was demonstrated to be a HCC prognostic marker, since increased p-ERK1/2 levels correlate with a decrease in the overall survival (35). MDA7/IL24 overexpression induces CCNE2 and p-ERK1/2 downregulation, indicating that this molecule suppresses tumor progression by regulating cell cycle transition. Just as demonstrated by the experimental results, IL-24 overexpression induced G1 arrest in human HCC cells.

BCL2 is a 26-kDa oncoprotein, and its carcinogenic property is closely associated with the anti-apoptotic activity (36). As a regulator of apoptosis, BCL2 may promote tumor cell survival and inhibit apoptosis through the regulation of mitochondrial membrane permeability and the induction of tumor angiogenesis (37). BCL2 overexpression is related to tumor progression (38). P-AKT, an active form of AKT, plays an important role in the inhibition of tumor cell apoptosis, promoting tumor cell proliferation and angiogenesis (39). Many studies demonstrated that p-AKT is highly activated in many tumors, and its abnormal expression was shown to be closely related to tumor development and progression (40). Members of caspase family are key elements in the process of apoptosis, and their activation and abnormal expression can induce apoptosis through the interaction with other factors (41). Caspase-3 is the most important member of the caspase family involved in the process of apoptosis, mediating signaling triggered by many other molecules (42). In the present study, the expression levels of p-AKT, BCL2, and caspase-3 were shown to differ between the cells overexpressing *MDA7/IL24* and the controls, indicating that MDA7/IL24 can inhibit tumor progression by inducing apoptosis, which is consistent with the results of cell functional experiments.

Taken together, our results demonstrate that MDA7/IL24 can inhibit the proliferation and suppress tumorigenicity of HCC cells *in vitro*. Furthermore, the MDA7/IL24 exerts its effects through the regulation of cell cycle transition and the induction of apoptosis. Therefore, we demonstrated that MDA7/IL24 has anticancer functions, its overexpression inhibits HCC progression, and it may represent a novel therapeutic target for cancer treatment.

Acknowledgements

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