ORIGINAL RESEARCH

Gene Therapy with Cytosine Deaminase and Endostatin Fusion Gene Mediated by Endothelial Progenitor Cells in Hepatomas

This article was published in the following Dove Press journal:
Cancer Management and Research

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Purpose: Gene-targeting therapy provides a novel therapeutic approach for tumor treatment using genetically modified endothelial progenitor cells (EPCs) as cellular carriers. This study applied EPCs armed with cytosine deaminase (CD) and endostatin (ES) fusion gene in liver cancer to explore its therapeutic effect.

Materials and Methods: EPCs from heart blood of male BALB/c nude mice were cultured and transfected with CD and ES fusion gene. Subsequently, these genetically modified cells were injected into mice bearing hepatoma through their tail veins. The tumor volumes and cell apoptosis were followed up.

Results: Tumor volume in the group injected CD/ES-EPCs greatly decreased. The positive rate of VEGF and CD31 in the tumor tissue was lowest in the CD/ES-EPC group. Furthermore, the number of apoptotic cells was highest in the CD/ES-EPC group.

Conclusion: The EPCs transfected with CD/ES inhibited tumor growth and preferentially induced tumor cell apoptosis, providing a novel methodology for cancer-targeting therapy.

Keywords: gene-targeting therapy, endothelial progenitor cell, endostatin, cytosine deaminase, hepatoma, transfection

Introduction

Hepatoma is the second leading cause of cancer-related deaths and one of the most common malignant tumors worldwide.[1,2] Although many surgical and nonsurgical treatments are available, eg liver transplantation, resection, transarterial chemoembolization, radio frequency ablation, and percutaneous ethanol injections,[3] the long-term prognosis remains poor.[4–6] Therefore, researchers are working on novel therapeutic approaches to improve the treatment outcomes of hepatoma patients.[7] Currently, gene-targeting therapy is potential candidate that can selectively attack the cancer by genetically-modified EPCs as cellular vectors.[8]

Endothelial progenitor cells (EPCs) play a decisive role in tumor vasculogenesis. Accumulating evidence indicates that EPCs migrate from the bone marrow into tumor tissues in vivo and differentiate into a new endothelium in the tumor bed, inducing the formation of new blood vessels.[9–11] EPCs can be used as cellular vehicles for cancer therapy.[8] EPCs represent a surrogate indicator for angiogenesis and response to antitumor strategies based on its mobilization.[12,13]

Endostatin (ES), initially discovered in the supernatant of a murine hemangioendothelioma cell line, was an inhibitor of endothelial cell proliferation in vitro and a robust angiogenesis inhibitor in vivo.[14] In addition, gene therapy of ES for

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hepatoma via inhibiting tumor angiogenesis provided a promising result.[15,16] However, there is no direct tumor-killing effect, which is a major drawback of these approaches.

Cytosine deaminase (CD) is a suicide enzyme capable of converting non-toxic 5-fluorocytosine (5-FC) into a cytotoxic 5-fluorouracil (5-FU), which is widely used as a chemotherapeutic agent.[17] Several clinical trials have demonstrated that CD has the capability to kill hepatoma cells.[18,19] In this study, the therapeutic effect of EPCs armed with CD and ES fusion gene were studied in mouse hepatoma models and the feasibility of this technique was evaluated as a potential therapeutic approach for hepatoma.

Materials and Methods
EPC Culture

Animal experimental procedure was approved by the Institutional Animal Care and Use Committee of Zhejiang University School of Medicine [No. SYXK (Zhejiang)2019-0012]. The EPCs were generated from the cardiac blood of male BALB/c nude mice, aged 6 weeks, provided by the Experimental Animal Center, Zhejiang University School of Medicine. The fresh blood withdrawn from the heart of the mice was heparinized and diluted with phosphate-buffered saline (PBS). Then, the monocyte layer was selected by density centrifugation and resuspended in microvascular growth medium-2 (EGM-2 MV; Cambrex, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS; SAFC Biosciences, St. Louis, MO, USA). Mononuclear cells (MNCs) were cultured in a 25 cm^3 culture flask with standard culture medium at 37 °C with 5% CO_2.

In vitro Transfection of EPCs

Lentiviral vectors (LVs) containing CD and ES fusion gene were constructed as described previously.[20] The LVs were constructed by transfection of 293T cells. The medium was changed after 24 h post-transfection. The conditioned medium collected after 48 h was centrifuged at 3000 rpm for 15 min and filter sterilized through 0.45 μm filters, followed by centrifugation at 50,000 $\times g$ for 90 min. The samples were diluted in DMEM and stored at −80 °C.

For infection, EPCs were plated 24 h before infection with LV-CD/ES (MOI＝5, 25, 50, 100 pfu/cell) in DMEM with 10% FBS for 48, 72, and 96 h at 37 °C in a CO_2 incubator. The transfection efficiency of EPCs was assessed using a fluorescence microscope. Quantitative real-time PCR was performed to detect the mRNA levels of CD and ES genes. Western blot analysis was performed to detect the protein concentrations of supernatants.

Proliferation Assay

An MTT-based colorimetric proliferation assay (Sigma, St. Louis, MO, USA) was employed to test the killing effect of the EPCs transfected with LV-CD/ES. Trypsinized and resuspended EPCs (3×10^4cells/mL) were plated in the fibronectin-coated 96-well plates (3×10^3/well) at 37 °C for 24 h. A volume of 10 μL MTT reagent was added to each well after washing with PBS, followed by incubation at 37 °C for an additional 3–4 h. The OD values were measured at 492 nm using an enzyme-linked immunosorbent detector (Thermo Scientific, Waltham, MA, USA).

Migration Assay

The chemotactic motility of the transfected EPCs was tested using Transwell chamber (Corning, USA) assay. A density of 1×10^5 cells, including EPCs, EPCs transfected with LVs, and EPCs transfected with LV-CD/ES were seeded in the upper chamber wells. The EGM-2 medium containing 10% FBS was placed in the lower chamber as a chemoattractant. The chamber was incubated at 37 °C for 48 h. After the non-migrating cells were discarded and upper wells washed with PBS, the filters were scraped with cotton swabs, and the cells were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet. The migrated cells were viewed and enumerated under an inverted microscope (Olympus).

Tumor Models and in vivo Antitumor Experiments

BALB/c nude mouse hepatoma model, aged 6 weeks, was provided and housed in specific-pathogen-free (SPF) laminar air flow rooms at the Experimental Animal Center, Zhejiang University School of Medicine. Animals had free access to food and water. All experiments in this study were performed in agreement with the experimental animal ethical standards of Zhejiang University School of Medicine.

The mouse tumor model of human HepG2 liver cancer was used in this study. HepG2 cells were purchased from Anti-Cancer Biotech Co., Ltd. (Beijing, China). An equivalent of 2×10^6 HepG2 cells (1 mL) suspension was injected into the subcutaneous tissues of the mice. Two

weeks post-implantation, a tumor of 3 cm was stripped from the subcutaneous tissues of the mice. The tumor was sliced into 1mm^3 fragments and injected into the mouse liver.

In order to assess the antitumor effects of CD/ES-EPCs in vivo, the mice were randomly assigned to five groups (n=15/group) as follows: group 1 normal mice without tumor, group 2 hepG2-bearing mice without treatment, group 3 hepG2-bearing mice with EPCs (injection via tail vein, 1×10^6 cells/mouse), group 4 hepG2-bearing mice with CD/ES (injection via tail vein, 1×10^8 TU/mL), and group 5 hepG2-bearing mice with CD/ES-EPCs (injection via tail vein, 1×10^6 cells/mouse). Next, 5-FC (500 mg/kg/d) was injected intraperitoneally into groups 4 and 5 two times daily after the last inoculation.

The Biospec 70/20USR 7.0T MRI (Bruker, Germany) was used for the detection of hepatoma in mice after in vivo transfection. The scanning parameters of MRI were as described previously:[20] T2WI scan; RARE sequence, TR/TE:1300 ms/7.5 ms; slice thickness 1 mm; images in acquisition 16; FOV 3.5 mm×3.5 mm; flip angle 160°; matrix 256×256. The volumes of the tumors were calculated by assessing the T2WI images using Render ToolKit 1.0.The mice in the study were sacrificed at the end of experiment.

Immunohistochemistry and Histological Staining

The tumors were excised and fixed in 10% formalin overnight. The tissues were dehydrated, embedded in paraffin, and sectioned into 5-µm slices for immunohistochemistry staining. The slides were blocked for the non-specific antibody, and subsequently, probed with primary antibodies overnight at room temperature. After rinsing with PBS, the slides were incubated with secondary antibodies for 60 min at ambient temperature and washed again with PBS. The immunostained sections from peripheral and central regions of each tumor were imaged using a fluorescent microscope (Olympus, Center Valley, PA, USA). A minimum of 5 fields/section and 5 sections/tumor sample were analyzed.

In vivo Apoptotic TUNEL (Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick End Labeling)

For apoptotic assay in vivo, tumor tissues were embedded in paraffin blocks. The slides with the sections were washed with PBS and incubated with TUNEL reaction mixture (Roche Diagnostics, USA) at 37 °C for 30 min according to the manufacturer's protocol. The apoptotic cells of positive TUNEL staining were counted in five random fields under a fluorescent microscope.

Statistical Analysis

Statistical analyses were performed using the SPSS® statistical package, version 25.0 (SPSS Inc., Chicago, IL, USA) for Windows®. Data for continuous variables are presented as means ± standard deviation (SD). The differences between the study and control groups were determined by the Mann–Whitney U-test or the unpaired Student's t-test where appropriate. P-values <0.05 were considered as statistically significant.

Results

In vitro Biological Function Assay of CD/ES

To inspect the possibility of CD/ES gene for cancer therapy, the mRNA levels of ES and CD genes were confirmed by quantitative real-time PCR. The protein expression of ES was confirmed by Western blot analysis. The results showed a significant increase in mRNA levels (Figure 1) and protein concentrations (Figure 2) in the groups transfected with CD/ES as compared to groups treated with EPCs or EPCs+LV.

To investigate the 5-FC prodrug-converting enzyme activity of CD/ES, the high-performance liquid chromatography (HPLC) assay was employed to measure the 5-FU concentrations of supernatants. This result showed a marked increase in 5-FU concentrations in the groups transfected with CD/ES (Figure 3).

Furthermore, to assess the antiangiogenic activity of CD/ES, we performed proliferation and migration assays to inspect the effects of the gene. The results showed a significant decrease in cell proliferation (Figure 4) and the number of migrated cells (Figure 5) in the groups transfected with CD/ES as compared to groups treated with EPCs or EPCs+LV.

In vivo Tail Vein-Injected CD/ES-EPCs Suppressed the Tumor Growth

After transplantation of CD/ES-EPCs plus 5-FC into the mouse with hepatoma, the tumor volumes among five groups (n=15/group) were observed by MRI. The results showed that the tumor volumes in the group transfected with CD/ES-EPCs plus 5-FC were smaller than that in the
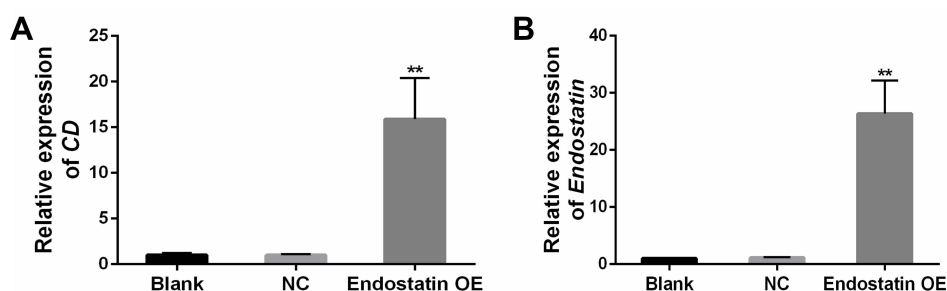
Figure 1 mRNA levels of CD and ES genes. (**A**) CD gene. (**B**) ES gene. Blank: EPCs; NC: EPCs transfected with LV; Endostatin OE: EPCs transfected with LV-CD/ES (******$P<0.01$ vs NC).
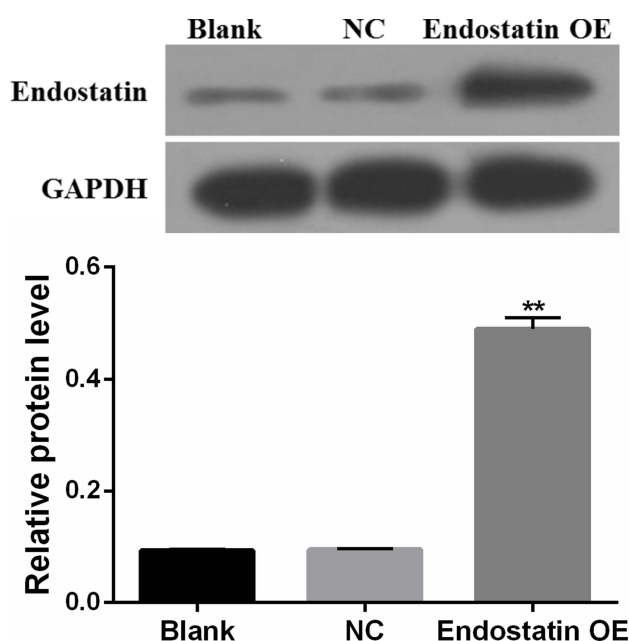

Figure 2 Western blot analysis of endostatin. Blank: EPCs; NC: EPCs transfected with LV; Endostatin OE: EPCs transfected with LV-CD/ES (**$P<0.01$ vs NC).
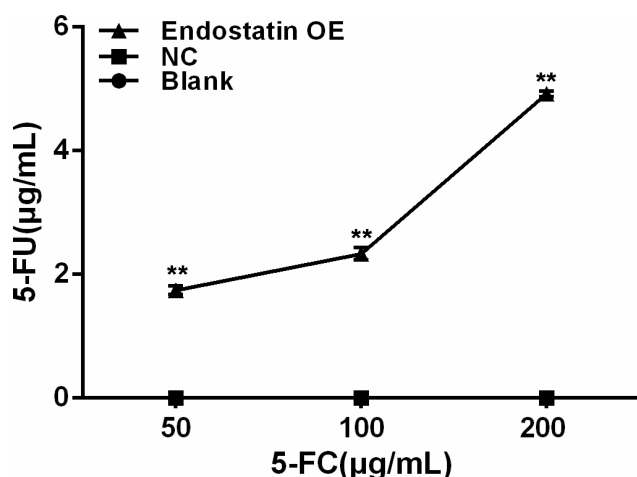

Figure 3 5-FU concentrations of supernatants. Blank: EPCs; NC: EPCs transfected with LV; Endostatin OE: EPCs transfected with LV-CD/ES (**$P<0.01$ vs NC).
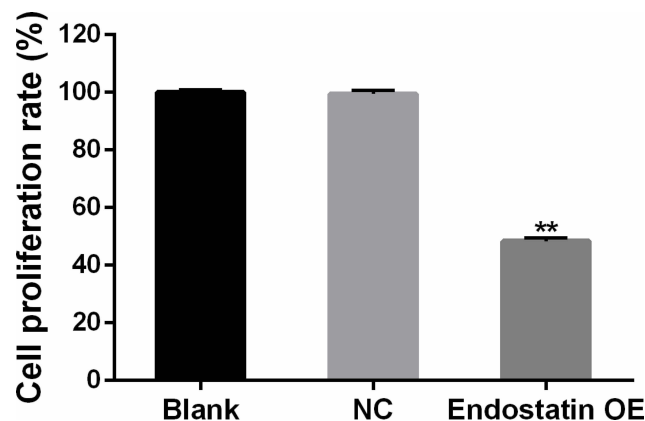
Figure 4 Cell proliferation among the three groups. Blank: EPCs; NC: EPCs transfected with LV; Endostatin OE: EPCs transfected with LV-CD/ES (**$P<0.01$ vs NC).

other groups receiving other treatments after 2 weeks post-transfection (Figure 6).

CD/ES-EPCs Inhibited Angiogenesis and Induced Tumor Cell Apoptosis

VEGF immunohistochemistry staining of the tumor tissues showed that the positive rate of VEGF in the tumor tissues was greatly reduced in the ES/CD-EPCs treated group when compared to the other treatment groups, (Figure 7). Similarly, the CD31-positive staining showed that the number of endothelial cells in the tumor tissues was greatly decreased in the ES/CD-EPCs treated group compared to the other treatment groups, further supporting the tumor suppression results (Figure 8). The number of apoptotic tumor cells detected by the TUNEL assay in the group treated with CD/ES-EPCs was significantly higher than that in other groups (Figure 9).

Discussion

CD is a therapeutic suicide gene for tumors and can convert the non-toxic 5-FC into cytotoxic 5-FU, which is a standard
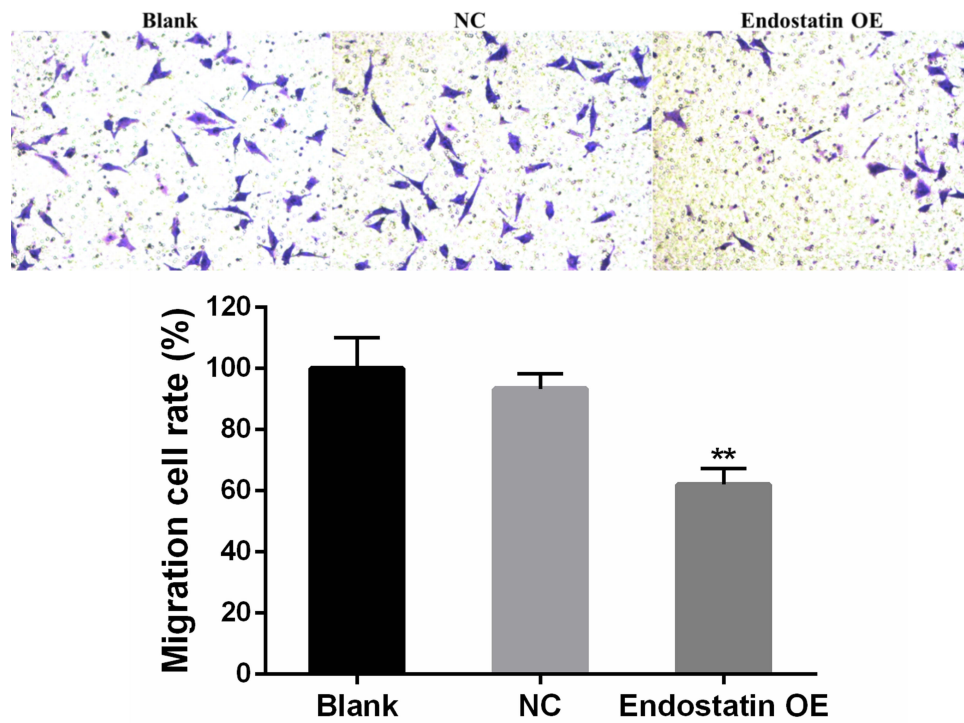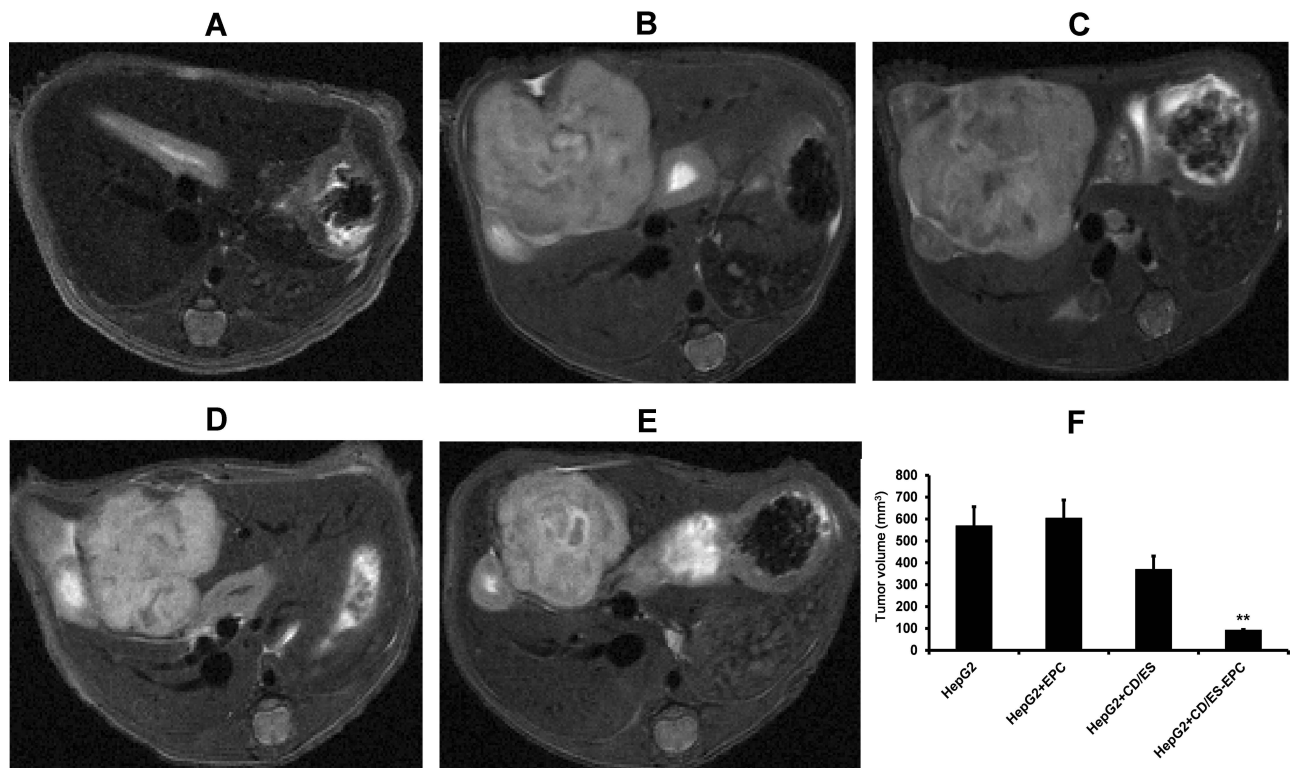
Figure 5 Cell migration among the three groups (×200). Blank: EPCs; NC: EPCs transfected with LV; Endostatin OE: EPCs transfected with LV-CD/ES (**P<0.01 vs NC).

Figure 6 Tumor volumes assessed by MRI after cell transplantation. (**A**) Control, (**B**) HepG2, (**C**) HepG2+EPC, (**D**) HepG2+CD/ES, (**E**) HepG2+CD/ES-EPC, and (**F**) statistical analysis of tumor volumes (**P<0.01 vs other groups).
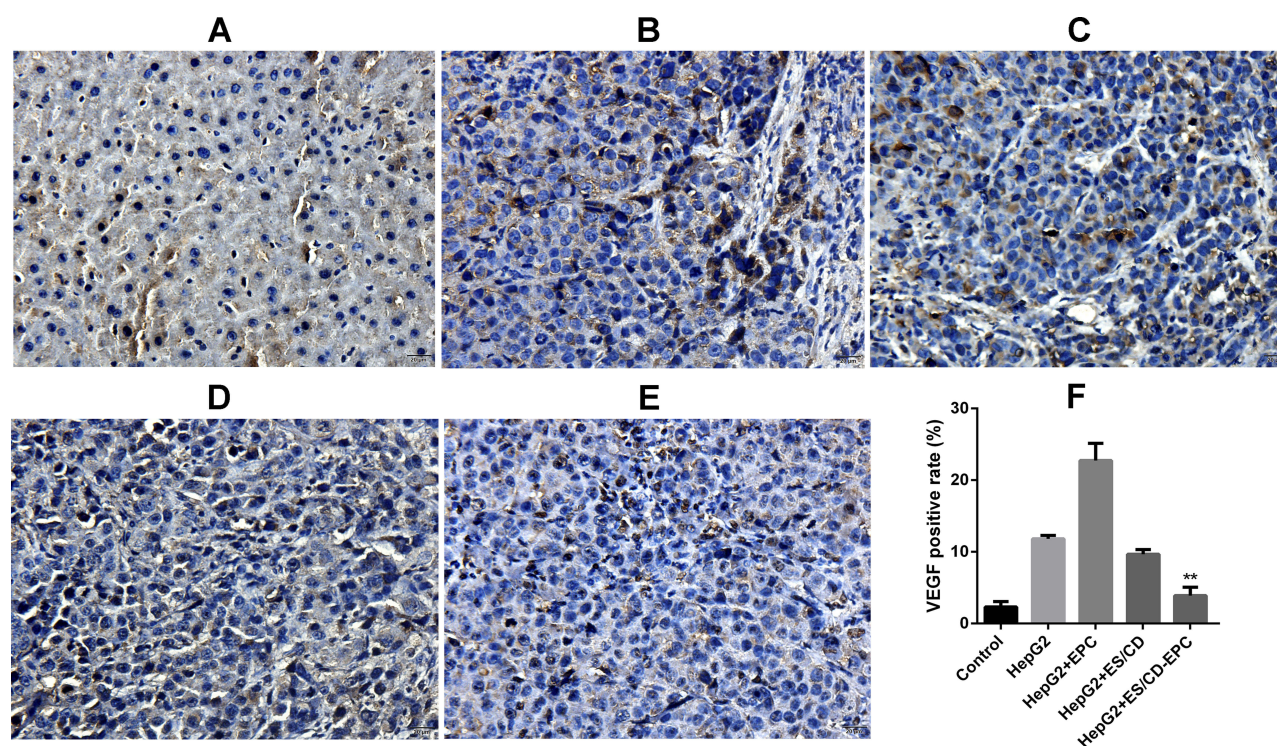
Figure 7 VEGF staining after cell transplantation. (**A**) Control, (**B**) HepG2, (**C**) HepG2+EPC, (**D**) HepG2+CD/ES, (**E**) HepG2+CD/ES-EPC, and (**F**) statistical analysis of VEGF-positive rate (***P*<0.01 vs other treatment groups).
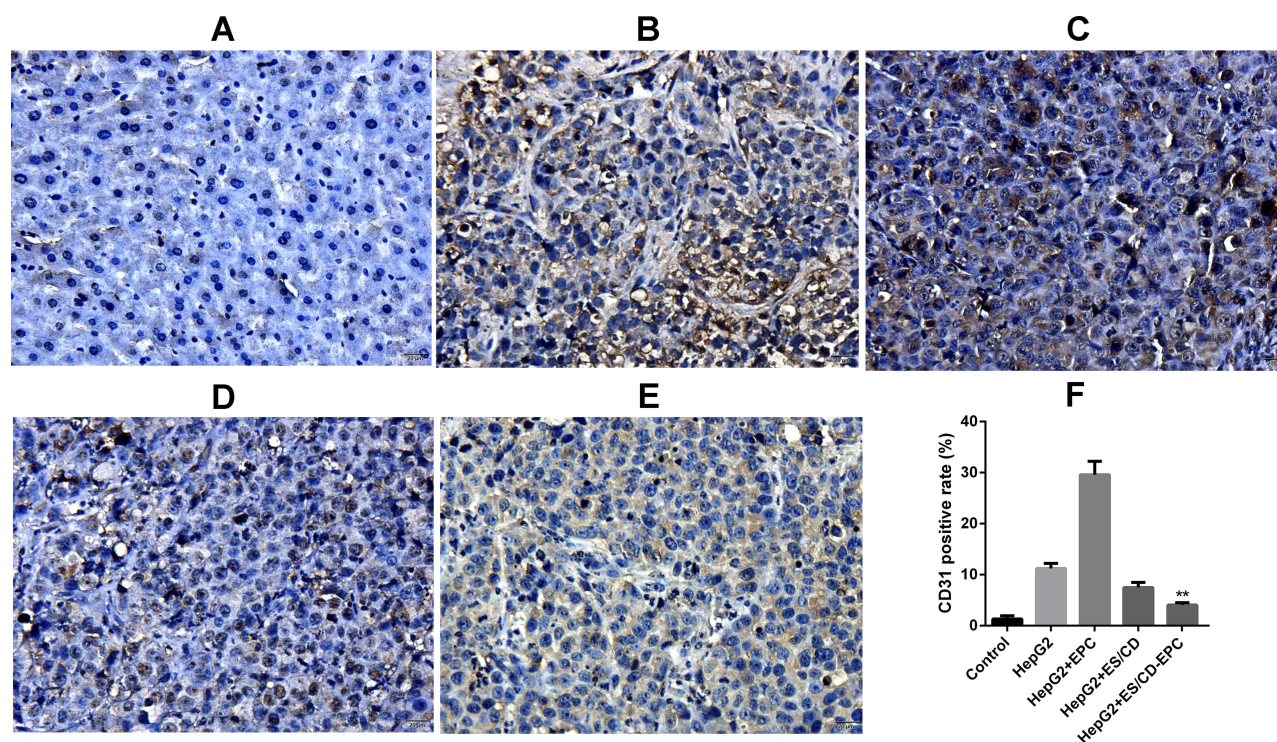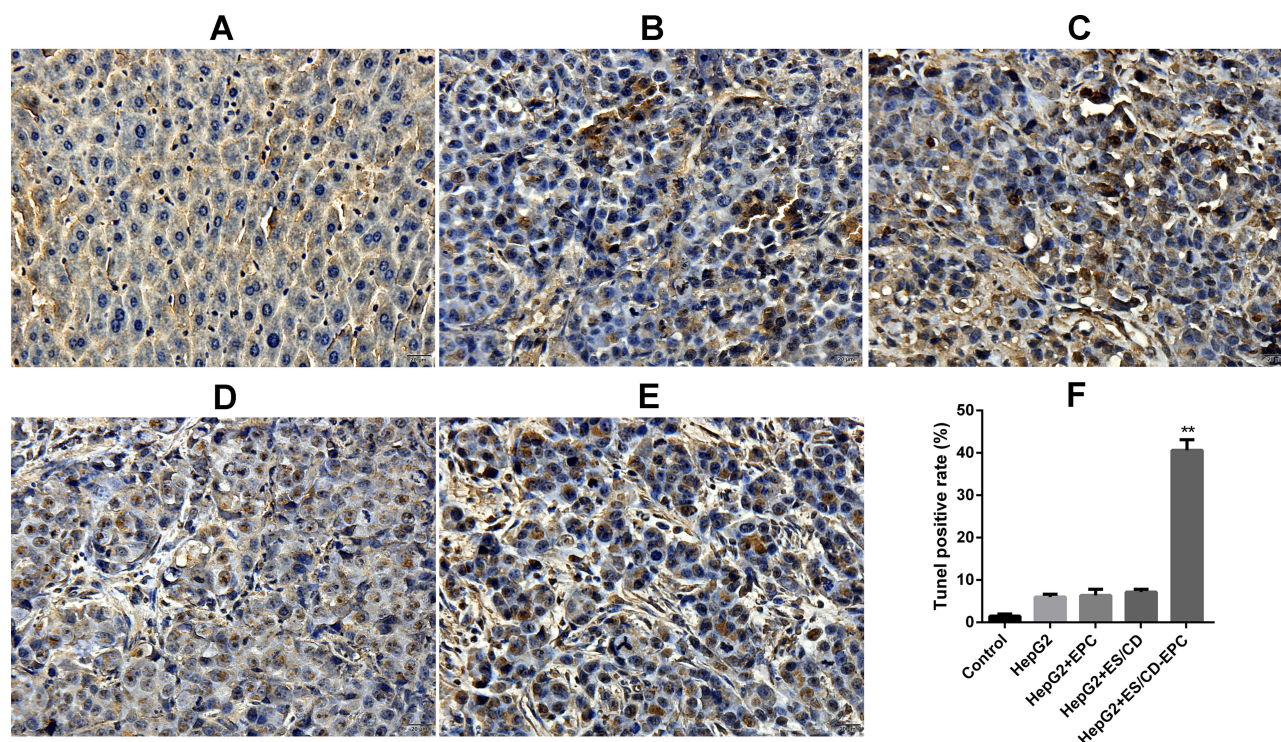

Figure 8 CD31-positive staining after cell transplantation. (**A**) Control, (**B**) HepG2, (**C**) HepG2+EPC, (**D**) HepG2+CD/ES, (**E**) HepG2+CD/ES-EPC, and (**F**) statistical analysis of CD31-positive rate (***P*<0.01 vs other treatment groups).

Figure 9 Apoptosis of tumor cells after cell transplantation. (**A**) Control, (**B**) HepG2, (**C**) HepG2+EPC, (**D**) HepG2+CD/ES, (**E**) HepG2+CD/ES-EPC, and (**F**) statistical analysis of TUNEL-positive rate (******$P<0.01$ vs other groups).

chemotherapeutic agent that inhibits RNA and DNA synthesis.[17] Metronomic chemotherapy suppressed the endothelial cell growth and induces apoptosis of tumor cell or cell growth arrest.[21] Similarly, ES can up-regulate several antiangiogenic genes, such as maspin, AT3, kininogen, vasostatin, and THBS-1. ES can also down-regulate several proangiogenic signaling pathway components, such as nuclear factor-β, activator protein-1, activators of transcription and signal transducers.[22] Recent studies have shown that ES suppresses the growth of tumor tissues in the tumor-bearing animal models.[20,23,24] The CD and ES genes act synergistically to effectuate the tumor-killing effect as well as inhibiting the tumor angiogenesis. In this study, the in vitro transfection of the CD/ES genes into the EPCs was confirmed by quantitative real-time PCR, Western blot analysis, and proliferation and migration assays.

Some studies have shown that the EPCs, predominantly homing to tumor tissues, might be used as cellular carriers for delivering the therapeutic signals to tumor sites. Varma et al used EPCs as part of a gene carrier/delivery system for glioma therapy and imaging probes. The study by Bonfim-Silva et al showed that the BM-derived EPCs are mobilized into the bloodstream, and specifically migrate to the tumor sites in vivo. To monitor the transplanted EPCs and verify the effectiveness of the EPCs as cellular carriers for tumor therapy, several non-invasive in vivo tracking imaging techniques such as MRI, nuclear medicine, and optical imaging have been used.[25,26] After transplantation of the intravenously administered CD/ES-EPCs into the mouse hepatoma models, MRI was used for in vivo observation of the volume changes of the tumors. In addition, the current in vivo study showed that the tumor volumes were reduced in the CD/ES-EPCs group as compared to the other treatment groups. Moreover, the VEGF staining and TUNEL test indirectly demonstrated that CD/ES-EPCs were present in the tumor tissues. The VEGF-positive rate was lowest and the number of apoptosis cells was highest in the CD/ES-EPCs group. This result illustrated that CD/ES-EPCs promote apoptosis of tumor cells and inhibit the tumor angiogenesis.

Conclusion

This study offers a novel treatment strategy for inhibiting the tumor growth by CD/ES-transfected EPCs targeting the tumor sites via vein grafting that can serve as a potent therapeutic option for the treatment of hepatoma after further investigation by clinical trials.

Ethical Statement

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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