

ORIGINAL ARTICLE

Lentivirus-mediated RASSF1A expression suppresses aggressive phenotypes of gastric cancer cells *in vitro* and *in vivo*

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Loss of Ras association domain family protein 1 isoform A (RASSF1A) expression is associated with the development of a variety of human cancers and the expression of carcinoembryonic antigen (CEA) frequently occurs in gastric cancer. This study investigated the effects of RASSF1A expression restoration using a hypoxia-inducible CEA promoter-driven vector on xenograft tumor growth in nude mice and on the *in-vitro* regulation of gastric cancer cell viability, cell cycle distribution, apoptosis, colony formation and invasion capacity. The data showed that the level of CEA mRNA and protein was much higher in gastric cancer SGC7901 cells than in a second gastric cancer cell line, MKN28, or in the MCF-10A normal epithelial breast cell line. RASSF1A expression was restored in SGC7901 cells compared with the negative control virus-infected SGC7910 cells. RASSF1A expression restoration significantly inhibited gastric cancer cell viability, colony formation and invasion capacity, but induced cell cycle arrest and apoptosis *in vitro*, especially under hypoxic culture conditions. At the gene level, restoration of RASSF1A expression under hypoxic culture conditions significantly suppressed matrix metalloproteinase-2 expression and prevented cyclinD1 expression. A nude mouse xenograft assay showed that the restoration of RASSF1A expression reduced gastric cancer xenograft formation and growth. In conclusion, the restoration of RASSF1A expression using a hypoxia-inducible and CEA promoter-driven vector suppressed aggressive phenotypes of gastric cancer cells *in vitro* and *in vivo*. These results suggest that LV-5HRE-CEAp-RASSF1A gene therapy may be a promising novel approach to treat advanced gastric cancer.

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INTRODUCTION

Gastric cancer is one of the most commonly diagnosed cancers and is the most frequent cause of cancer death in the world.^{1,2} Although there have recently been significant advancements in gastric cancer treatment, including new surgical, chemotherapeutic and radiotherapeutic methods, many patients are still dying after being diagnosed with advanced gastric cancer and developing metastasized disease.³ Thus, gastric cancer remains a major clinical challenge. Tumor-targeted gene therapy may be a promising new approach for the effective control of gastric cancer. Gene therapy is defined as the use of vectors to bring nucleic acids into cells to alter gene expression and prevent, halt or reverse a pathological process. The number of ongoing or completed gene therapy clinical trials approved worldwide since 1996 has reached 1274 and 63.8% of these were for human cancer.⁴ Although significant progress has been made in the development and use of tumor-selective delivery systems in the past 20 years, there are still many challenges that must be overcome to develop therapeutic genes that are specifically expressed in cancer cells, but not in normal cells. To achieve this goal, strategies have been developed that include the use of specific gene promoters, enhancers and 5'-untranslated region that respond to tumor-specific transcription factors, to drive or knock down gene expression to treat human cancers.

We focused on the Ras association domain family protein 1 isoform A (RASSF1A), because lost RASSF1A expression is associated with the development of a variety of human cancers, including gastric cancer or other gastrointestinal malignancies, and this suggests the possibility that RASSF1A may have a tumor

suppressor function in these human cells.^{5–7} Restoration of RASSF1A expression, therefore, could potentially control gastric cancer progression. Moreover, carcinoembryonic antigen (CEA) is a glycosylated protein that is also highly expressed in a number of cancers, including gastric cancer.⁸ The CEA promoter has been used in many cancer studies and can specifically target CEA-positive cancer cells.^{9,10} Hypoxia-inducible factors (HIFs) are another potential target. Hypoxia frequently occurs in solid tumor lesions.¹¹ Accumulating data shows that 50%–60% of locally advanced solid tumors develop hypoxia.¹² Hypoxia promotes tumor progression, invasion of adjacent tissues and metastasis, and hypoxic tumors are resistant to radiotherapy and chemotherapy.¹³ HIF-1, a well-studied HIF, is a heterodimer of the HIF-1 α and HIF-1 β subunits. One subunit, HIF-1 α , is an O₂-regulated subunit that induces HIF-1 activity and expression in response to a reduction of O₂ availability, whereas the other, HIF-1 β , is a constitutive subunit.¹⁴ HIF-1 α binds to the hypoxia-responsive element (HRE), a conserved transcription promoting sequence in the regulatory regions of target genes.¹⁵ Previous studies have shown that HIF-1 α contributes to the malignant phenotypes of gastric cancer.^{16–18}

In this study, to restore RASSF1A expression, we constructed an expression vector carrying RASSF1A under the control of hypoxia-inducible and CEA promoters, to specifically restore RASSF1A expression in gastric cancer cells. We then assessed the effects of RASSF1A expression on xenograft tumor growth in nude mice and the regulation of gastric cancer cell viability, cell cycle distribution, apoptosis, colony formation and invasion capacity *in vitro*. The results of this study could help the future development of an

inducible tumor-specific lentiviral vector that combines RASSF1A with CEAp and 5HRE (LV-5HRE-CEAp-RASSF1A) for gastric cancer gene therapy.

RESULTS

Expression of endogenous CEA and RASSF1A mRNA and protein in gastric cancer cells

We first detected expression of endogenous CEA mRNA using quantitative reverse-transcription PCR in several gastric cancer cell lines as well as in a normal breast cell line. The results showed that the level of CEA mRNA in gastric cancer SGC7901 cells was much higher than that in both the MKN28 gastric cancer and normal breast MCF-10A cell lines (Figure 1a). The same was true for endogenous CEA protein (Figure 1b). The mean density of the CEA protein, as quantified using the immunohistochemical staining results, was 0.111 ± 0.024 , 0.076 ± 0.016 and 0.002 ± 0.000 in SGC7901, MKN28 and MCF-10A cells, respectively ($P < 0.001$ between SGC7901 or MKN28 and MCF-10A cells; $P < 0.01$ between SGC7901 and MKN28 cells). These data clearly indicate that the level of endogenous CEA protein was highest in SGC7901 cells, moderate in MKN28 cells and lowest in MCF-10A cells. Furthermore, endogenous RASSF1A protein was not expressed in SGC7901 or MKN28 cells, but was expressed in MCF-10A cells (Figure 1c). Thus, due to the levels of CEA and RASSF1A expression, we selected SGC7901 cells (CEA mRNA+, CEA protein+, RASSF1A protein-) for the experimental group and MKN28 cells (CEA mRNA-, CEA protein+, RASSF1A protein-) for the negative control.

Restoration of RASSF1A expression in gastric cancer cells using a CEAp or 5HRE-driven expression vector

We stably restored RASSF1A expression in gastric cancer cells using CEAp or 5HRE-driven expression vectors. RASSF1A expression in SGC7901/5HC-RASSF1A and MKN28/5HC-RASSF1A cells was confirmed by quantitative reverse-transcription PCR and western blotting (Figures 2a and b). SGC7901/5HC-RASSF1A cells showed very high levels of RASSF1A mRNA under hypoxic conditions ($300 \mu\text{M l}^{-1}$ cobalt chloride (CoCl_2)), but only slightly elevated levels of RASSF1A mRNA under normoxic conditions. Similarly, RASSF1A protein levels were significantly increased under hypoxic conditions. However, RASSF1A expression was low

in SGC7901, SGC7901/NC, MKN28/5HC-RASSF1A and MKN28 cells under both normoxic and hypoxic conditions. These data indicated that both RASSF1A mRNA and protein were expressed in SGC7901/5HC-RASSF1A cells, especially under hypoxic conditions.

The hypoxic inducibility, CEA-targeting and RASSF1A expression-driving ability of the 5HRE-CEAp element was confirmed using a dual luciferase assay (Figure 2c). SGC7901, MKN28 and MCF-10A cells were transfected with pGL4.20-5HRE-CEAp-Luc or control vectors and cultured for 24 h under normoxic or hypoxic conditions. The luciferase activity fold change was measured and the data showed a significant increase in luciferase activity in SGC7901 cells under normoxic conditions ($P < 0.05$; 59.63 ± 24.24 in SGC7901 vs 27.82 ± 7.19 in MKN28 and 1.25 ± 0.57 in MCF-10A). However, luciferase activity in SGC7901 and MKN28 cells was further increased in the presence of CoCl_2 ($300 \mu\text{M l}^{-1}$) when compared with luciferase activity under aerobic conditions (SGC7901, $P < 0.001$; 519.75 ± 97.14 vs 59.63 ± 24.24 ; MKN28 $P < 0.001$; 87.80 ± 7.78 vs 27.82 ± 7.19).

Effects of RASSF1A expression on regulation of gastric cancer cell viability, apoptosis, cell cycle distribution, colony formation and invasion capacity *in vitro*

The viability of cells expressing RASSF1A was assessed using a CCK-8 assay. The data showed that RASSF1A expression significantly reduced SGC7901/5HC-RASSF1A cell viability when compared with either SGC7901 or SGC7901/NC cell viability under both normoxic and hypoxic conditions. Under hypoxic conditions, RASSF1A expression further decreased tumor cell viability (Figures 3a and b). However, this result was not replicated in MKN28/5HC-RASSF1A cells (Figure 3b), because MKN28 cells do not express CEA and, thus, the vector did not restore RASSF1A expression in these cells (Figure 2b).

Moreover, a tumor cell apoptosis assay showed that the rate of apoptosis was increased in SGC7901/5HC-RASSF1A cells under hypoxic conditions (Figure 4a). Cell cycle analysis revealed that SGC7901/5HC-RASSF1A cells accumulated at the G1 phase of the cell cycle under hypoxic, but not normoxic, conditions ($P < 0.05$; Figure 4b). Colony formation assays also showed a significant reduction in the numbers of colonies that were formed on the plate by SGC7901/5HC-RASSF1A cells under hypoxic conditions ($P < 0.05$; Figures 5a and b). In addition, the Transwell assay showed that the number of tumor cells passing through the

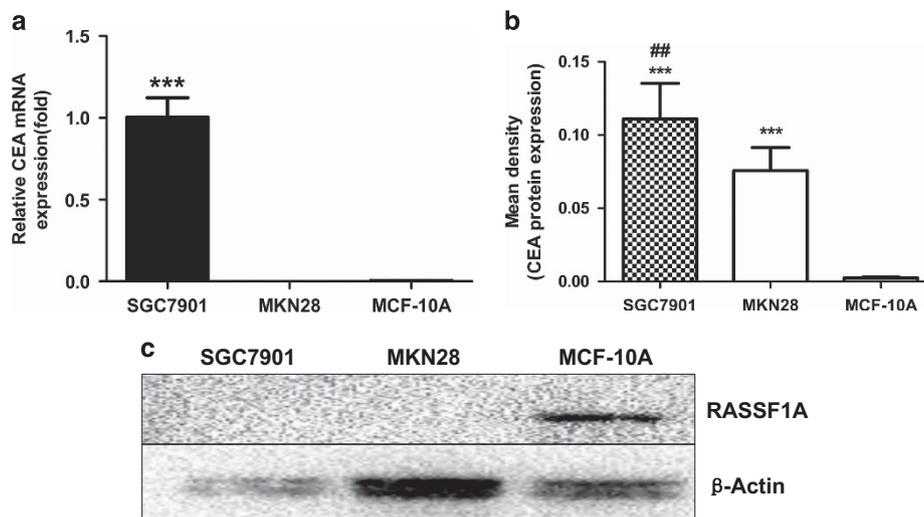


Figure 1. Expression of endogenous CEA and RASSF1A mRNA or protein in SGC7901, MKN28 and MCF-10A cells. **(a)** Quantitative reverse-transcription (qRT-PCR) analysis of CEA mRNA levels $***P < 0.001$ vs MKN28 and MCF-10A. **(b)** Quantified data for CEA protein levels in SGC7901, MKN28 and MCF-10A cells. **(c)** Western blot analysis of RASSF1A protein levels in SGC7901, MKN28 and MCF-10A cells. $***P < 0.001$ vs MCF-10A; $**P < 0.01$ vs MKN28.

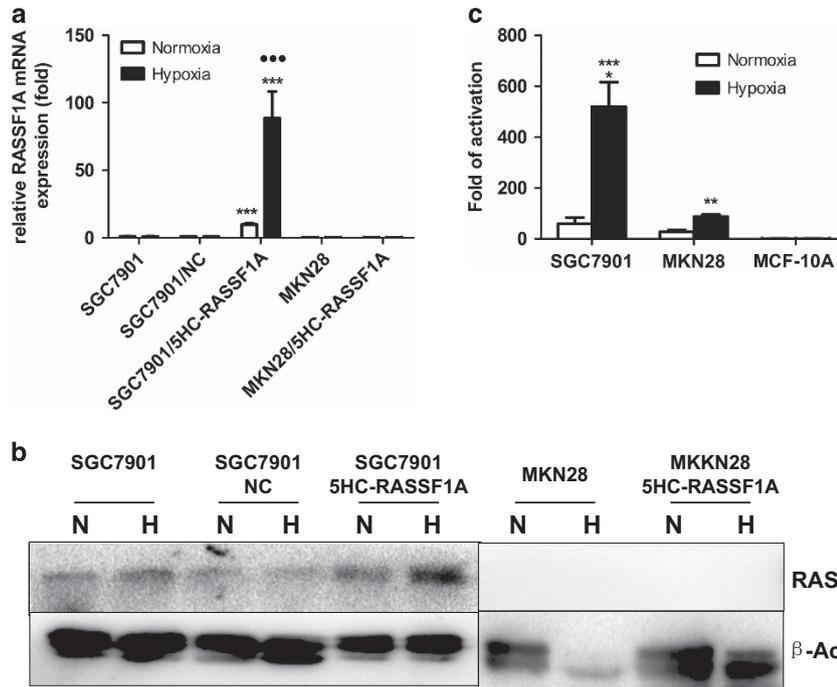


Figure 2. Overexpression of RASSF1A mRNA in SGC7901 and MKN28 cells. **(a)** Quantitative reverse transcription (qRT-PCR) analysis of RASSF1A mRNA in RASSF1A stably transfected cells. $***P < 0.001$ vs SGC7901, SGC7901/NC, MKN28 and MKN28/5HC-RASSF1A cells; $***P < 0.001$ vs SGC7901/5HC-RASSF1A cells cultured under normoxic conditions. **(b)** Western blot analysis of RASSF1A protein in RASSF1A stably transfected cells under normoxic (N) and hypoxic (H) conditions. **(c)** Luciferase activity assay. 5HRE-CEAp activity was measured in SGC7901, MKN28 and MCF-10A cells. $*P < 0.05$ vs MKN28 and MCF-10A cells under hypoxic conditions; $**P < 0.001$ vs MKN28 cells under normoxic conditions; $***P < 0.001$ vs SGC7901 under normoxic conditions.

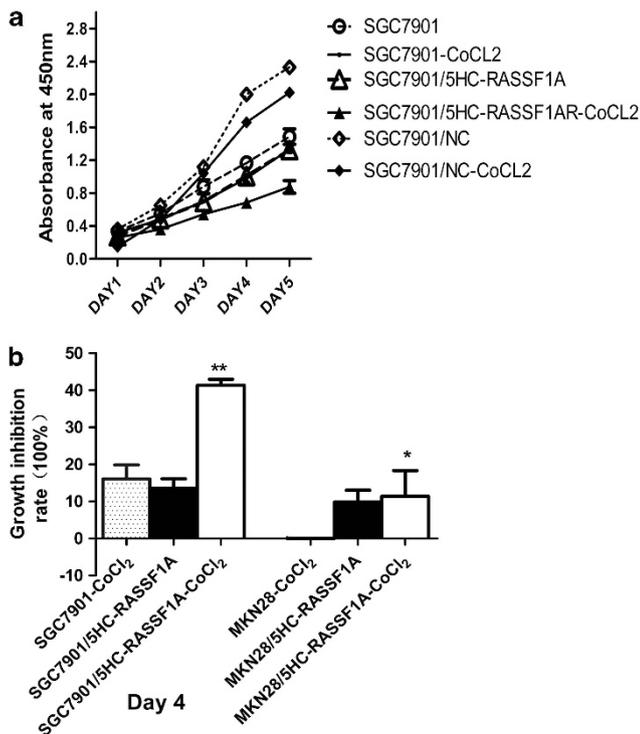


Figure 3. Effects of RASSF1A expression on SGC7901 and MKN28 cell viability. **(a)** Cell viability CCK-8 assay. SGC7901, SGC7901/NC and SGC7901/5HC-RASSF1A cells were grown in monolayer with or without CoCl_2 for 5 days and then subjected to cell viability analysis. **(b)** Growth inhibition of SGC7901, SGC7901/5HC-RASSF1A, MKN28 and MKN28/5HC-RASSF1A cells with or without CoCl_2 on the fourth day after treatment. $**P < 0.01$ vs SGC7901/5HC-RASSF1A and SGC7901- CoCl_2 ; $*P > 0.05$ vs MKN28/5HC-RASSF1A.

Matrigel-coated membrane was significantly reduced in the SGC7901/5HC-RASSF1A cells ($P < 0.01$) and was further reduced under hypoxic conditions (Figures 5c and d), suggesting that RASSF1A expression suppressed gastric cancer cell invasion.

Effects of RASSF1A expression on the regulation of the HIF-1 α , caspase 3, MMP-2 and cyclinD1 proteins in gastric cancer cells

To investigate the molecular events associated with recombinant lentivirus vector pLV-5HRE-CEAp-RASSF1A-mediated RASSF1A expression in gastric cancer cells, we assessed whether restoring RASSF1A expression under hypoxic conditions could affect the expression of HIF-1 α , caspase 3, matrix metalloproteinase-2 (MMP-2) and cyclinD1 proteins in SGC7901 cells. HIF-1 α protein was not detected in SGC7901, SGC7901/NC or SGC7901/5HC-RASSF1A cells under normoxic conditions; however, expression could be equally detected in all cell lines under hypoxic conditions (Figure 6a). Conversely, MMP-2 protein levels were significantly reduced under hypoxic conditions ($P < 0.01$; Figures 6a and b). Although caspase 3 protein showed no expression differences in SGC7901, SGC7901/NC or SGC7901/5HC-RASSF1A cells under normoxic conditions, its expression only increased under hypoxic conditions in SGC7901/5HC-RASSF1A cells ($P < 0.01$; Figures 6a–c). CyclinD1 expression was increased under hypoxic conditions when compared with normoxic conditions, but RASSF1A expression prevented cyclinD1 expression under hypoxic conditions ($P < 0.01$; Figures 6a–d).

Effect of RASSF1A on gastric cancer xenograft formation and growth in nude mice

SGC7901 cells were subcutaneously implanted in BALB/c nude mice. Xenograft tumors formed within 8 days and grew to 50–100 mm^3 . We injected lentiviruses (1×10^8 pfu) in 100 μl of serum-free medium intratumorally at three sites per xenograft tumor and repeated the injections on day 4. Two weeks after the

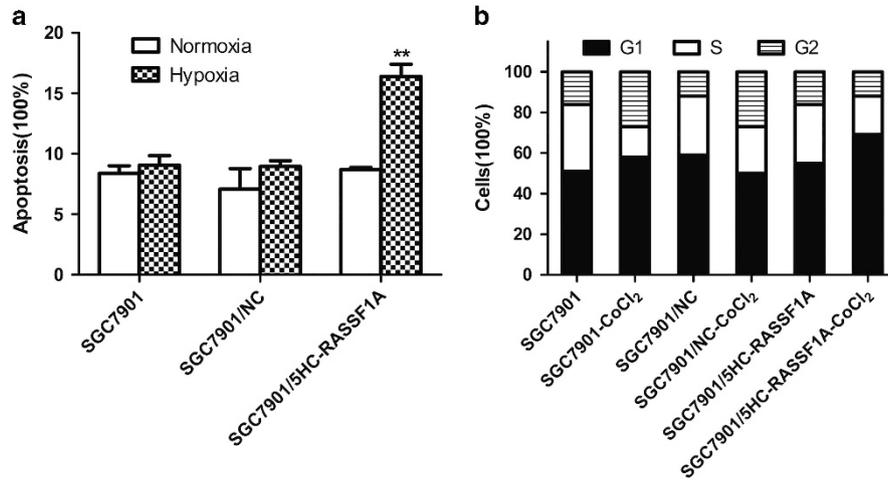


Figure 4. Effect of RASSF1A expression on the regulation of SGC7901 cell apoptosis and cell cycle distribution under normoxic or hypoxic conditions. **(a)** Flow cytometry apoptosis assay. SGC7901 cells were grown and subjected to tumor cell apoptosis analysis. $**P < 0.01$ vs SGC7901 and SGC7901/NC cells under normoxic or hypoxic conditions and SGC7901/5HC-RASSF1A cells under normoxic conditions. **(b)** Flow cytometric cell cycle analysis. Cells were grown under normoxic or hypoxic conditions and subjected to cell cycle analysis.

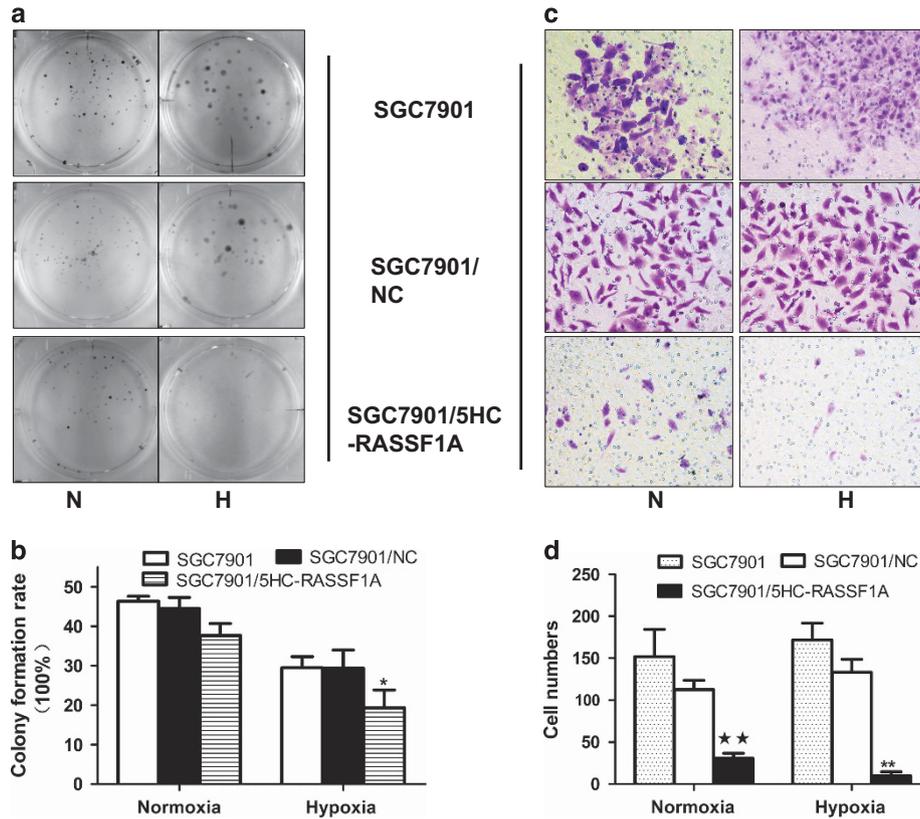


Figure 5. Effect of RASSF1A expression on the regulation of tumor cell colony formation and migration capacity in normoxic (N) or hypoxic (H) conditions. **(a)** Colony formation assay. SGC7901, SGC7901/NC and SGC7901/5HC-RASSF1A cells were grown under normoxic or hypoxic conditions and subjected to colony formation assay. **(b)** Summarized data of **a**. $*P < 0.05$ vs SGC7901 or SGC7901/NC cells under hypoxic conditions. **(c)** Transwell tumor cell migration assay. SGC7901, SGC7901/NC and SGC7901/5HC-RASSF1A cells were grown under normoxic or hypoxic conditions and subjected to a tumor cell migration assay. **(d)** Summarized data of **c**. $**P < 0.01$ vs SGC7901 or SGC7901/NC cells under normoxic conditions; $**P < 0.01$ vs SGC7901/5HC-RASSF1A cells under normoxic conditions.

second virus injection, mice were killed and xenograft tumors were collected, measured and photographed. Compared with the negative solution and LV-NC (pLV-5HRE-CEAp-NC) groups, the size and weight of the tumors were significantly lower in the LV-5HC-RASSF1A-treated mice ($P < 0.01$; Figure 7).

Immunohistochemical data also confirmed RASSF1A expression in gastric cancer cell xenografts. As shown in Figure 7d, RASSF1A expression was lower in the negative solution-injected or LV-NC-injected xenograft tumors, but RASSF1A expression was restored in xenograft tumors after the LV-5HRE-CEAp-RASSF1A injection. It

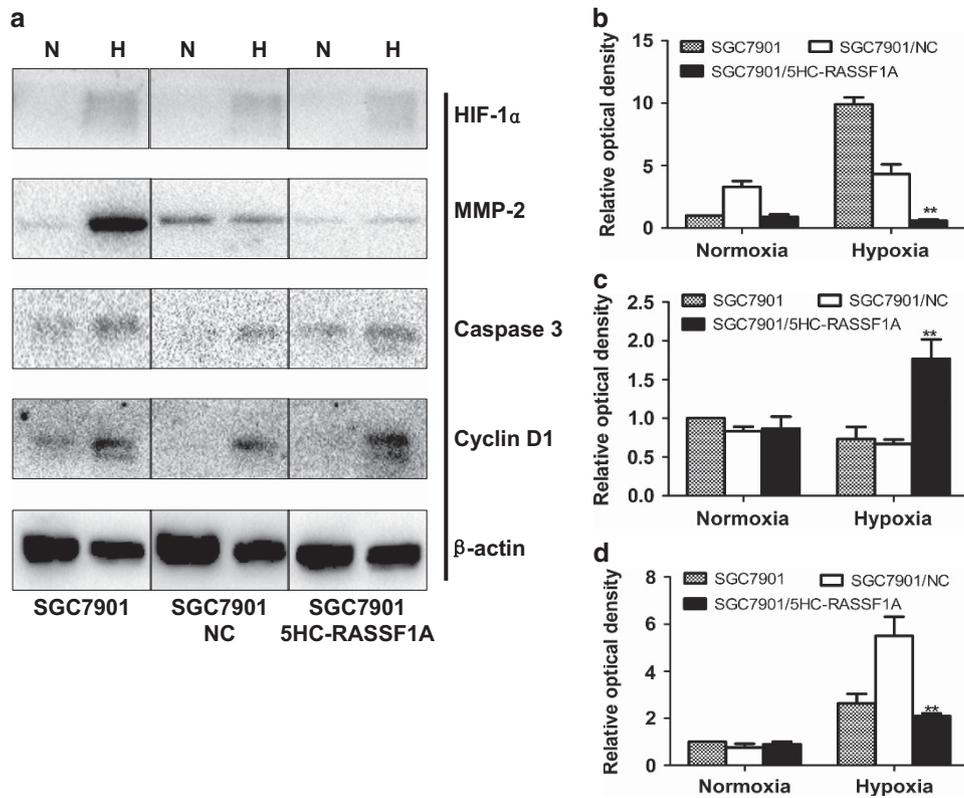


Figure 6. Effect of RASSF1A expression on the regulation of HIF-1 α , caspase 3, MMP-2 and cyclinD1 protein expression in SGC7901 cells under normoxic (N) or hypoxic (H) conditions. (a) Western blot analysis of HIF-1 α , caspase 3, MMP-2, cyclinD1 and β -actin in SGC7901, SGC7901/NC and SGC7901/5HC-RASSF1A cells. β -Actin was used as the internal control. (b) Summarized data of MMP-2 protein in cells. $**P < 0.01$ vs SGC7901 or SGC7901/NC cells under hypoxic conditions and SGC7901/5HC-RASSF1A cells under normoxic conditions; (c) Summarized data of caspase 3 protein in cells. $**P < 0.01$ vs SGC7901 or SGC7901/NC cells under hypoxic conditions; (d) Quantification of cyclinD1 protein expression in cells, $**P < 0.01$ vs SGC7901/5HC-RASSF1A cells under normoxic conditions.

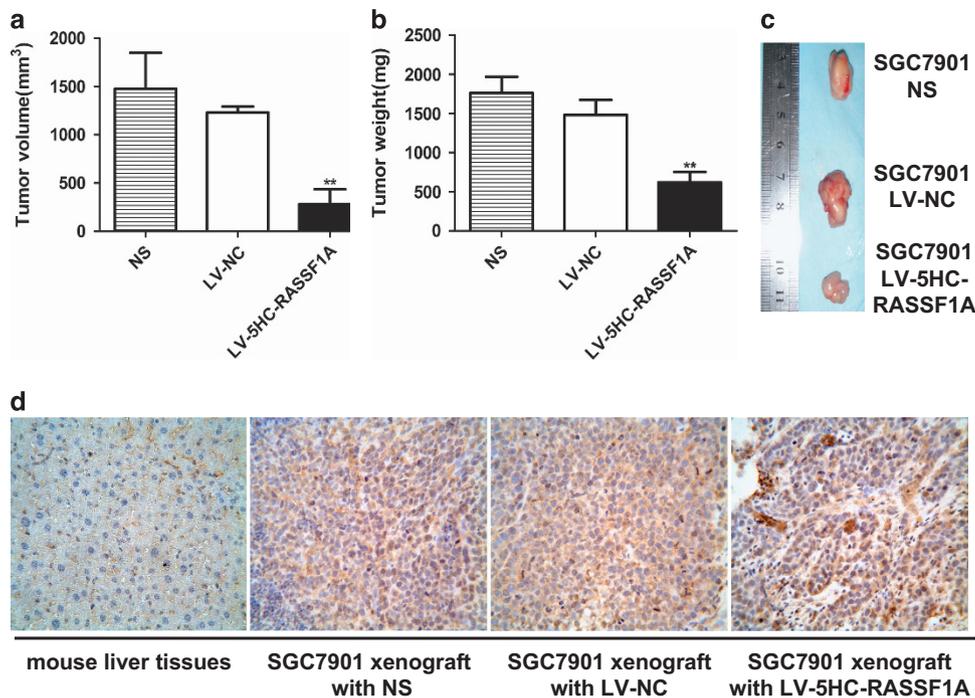


Figure 7. Effect of RASSF1A expression on the regulation of tumorigenicity in nude mice. (a) The mean tumor volume at the end of experiments. $**P < 0.01$ vs NS and LV-NC groups. (b) The mean tumor weight at the end of experiments. $**P < 0.01$ vs NS and LV-NC groups. (c) Image of xenograft tumors from each group. (d) Immunohistochemical analysis of RASSF1A expression in xenograft tumors and normal mouse liver tissues.

is also true that there was no RASSF1A expression in normal mouse hepatocytes in the liver tissues (Figure 7d).

DISCUSSION

Gene therapy is a promising option for the treatment human cancers; however, two keys to success, a tumor-selective delivery system and the persistent expression of anti-cancer gene products, remain elusive. In the current study, we developed a lentivirus vector for hypoxia-inducible, CEA promoter-driven and tissue-specific expression of RASSF1A, LV-5HRE-CEAp-RASSF1A. Assessing this vector showed that the following: (i) the regulatory element 5HRE-CEAp can induce luciferase reporter activity under hypoxic conditions in gastric cancer SGC7901 cells expressing high levels of CEA; (ii) RASSF1A expression levels were induced in RASSF1A-negative gastric cancer SGC7901 cells under hypoxic conditions; (iii) the LV-5HRE-CEAp-RASSF1A lentivirus suppressed aggressive SGC7901 gastric cancer cell phenotypes under hypoxic conditions, including cell viability, apoptosis, cell cycle arrest, colony formation and invasion capacity; (iv) RASSF1A expression regulated the expression of the caspase 3, MMP-2 and cyclinD1 proteins; and (v) LV-5HRE-CEAp-RASSF1A infection suppressed gastric cancer xenograft tumor formation and growth in nude mice. Thus, our current study provided a gene (RASSF1A is frequently lost in gastric cancer or other gastrointestinal cancers) and specific promoter-driven RASSF1A expression (CEA is usually overexpressed in these cancers) for future control of gastric cancer. Thus, our current data strongly supports the potential usefulness of LV-5HRE-CEAp-RASSF1A as an effective anti-tumor treatment option for advanced gastric cancer.

A tumor-selective delivery system is the key to successful tumor gene therapy. Over time, many gene therapy strategies have been developed and these strategies have been accompanied and shaped by an increasing knowledge of tumor pathogenesis. Hypoxia, caused by rapid tumor growth, has a key role in cancer progression and is the focus of many cancer treatment strategies.¹⁹ For example, Shibata *et al.*²⁰ constructed an NTR/CB1954 vector under the control of five copies HRE and a minimal cytomegalovirus promoter (CMV) promoter (5HRE/CMVmp) and confirmed specific anti-tumor activity under hypoxic conditions. They showed that among various HRE constructs and promoter elements, 5HRE/CMVmp had optimal activation at a low oxygen tension, which is true in the gastrointestinal tract. Harvey *et al.*²¹ developed an adenovirus vector for hypoxia-targeted gene therapy using the herpes simplex virus thymidine kinase and bacterial nitroreductase prodrug-activating genes. They showed that 5HRE derived from vascular endothelial growth factor and linked to the CMV minimal promoter could induce optimum luciferase reporter gene expression.

In the current study, we constructed a lentiviral vector under the control of a 5HRE element and a CEA promoter to enhance RASSF1A expression in tumor lesions. This 5HRE regulatory element showed hypoxia-inducible activity and its design conformed to the HRE described by Shibata *et al.*²⁰ In addition to a hypoxia-driven strategy for tumor targeting, the inclusion of the CEA promoter serves as a secondary tumor targeting strategy. The CEA promoter has been used in many studies, is highly expressed in a number of tumors and is a known tumor marker of digestive cancer. Nyati *et al.*²² used the CEA promoter and enhancer to selectively drive prodrug yeast cytosine deaminase expression in CEA-positive cancer cells and demonstrated that their vector had the ability to suppress tumor growth. Liu *et al.*²³ constructed a CMV enhancer and CEA promoter regulatory element, and demonstrated the ability to specifically target CEA-positive gastric cancer. Although the CEA promoter was used as part of a strategy of specific cancer gene delivery, the CEA promoter alone could only weakly drive target transgene expression, leading to insufficient anti-cancer gene expression

for cancer gene therapy.²⁴ To overcome this challenge, various enhancers were added in front of the CEA promoter.^{22–24} We used 5HRE as the CEA promoter enhancer.

Although it has only been studied for 14 years, RASSF1A is reported to be a putative tumor suppressor gene. Dammann *et al.*²⁵ showed that RASSF1A is localized at chromosome 3p21.3 and that it was inactivated by methylation of the CpG-island promoter. They also showed that RASSF1A expression was lost in lung tumors and re-expression of RASSF1A had anti-oncogenic effects in lung carcinoma cells. Since then, accumulating data has demonstrated that through multi-mechanisms, RASSF1A is a tumor suppressor gene in the vast majority of human cancers.²⁶ Our data, reported here, also demonstrated that RASSF1A has effective anti-tumor activity. Shivakumar *et al.*²⁷ showed that RASSF1A expression blocked cell cycle progression at the G1/S phase by inhibiting cyclinD1 accumulation. Agathangelou *et al.*²⁸ found that transfection of RASSF1A cDNA into A549 cells induced cell cycle arrest at the G1 phase and downregulated cyclinD1 expression. Our current study further confirmed these results and showed that the effects were significantly increased under hypoxic culture conditions. However, the cell cycle impact of RASSF1A under hypoxic conditions may be due to other unknown mechanisms. Apoptosis promotion is an important tumor suppressor function of RASSF1A and many studies have investigated the pro-apoptotic mechanisms of RASSF1A.^{29–32} Our current study demonstrated the effect of RASSF1A on the induction of SGC7901 apoptosis under hypoxic conditions. Furthermore, other studies have demonstrated that RASSF1A can suppress the migration of tumor cells.³³ Our current study revealed that the mechanism by which RASSF1A inhibits the invasive capacity of SGC7901 cells under hypoxic conditions might be related to the inhibition of MMP-2 expression. Although hypoxia can induce tumor progression, our current data indicate that LV-5HRE-CEAp-RASSF1A can effectively use that same hypoxia to drive the anti-tumor activity of RASSF1A.

Our current study is just 'proof-of-principle' and much more work must be done before our results can be translated into clinical trials, such as safety and efficiency of this lentivirus. Our LV-5HRE-CEAp-RASSF1A system may help to solve two key gene therapy problems in cancer, specificity and efficiency. We showed that this system can induce gene expression in CEA-positive gastric cancer cells under hypoxic conditions, further ensuring the cancer cell-specific expression of therapeutic genes. In addition, this study represents the first successful use of the tumor suppressor gene RASSF1A in gene therapy and confirms that the expression of RASSF1A effectively inhibits the growth of gastric cancer cells *in vivo* and *in vitro*. More work should be done in the future to verify the persistence and specificity of RASSF1A gene expression using the system described in this study.

MATERIALS AND METHODS

Cell lines and cultures

All cell lines were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Human gastric adenocarcinoma cell lines (SGC-7901 and MKN28) were grown in RPMI1640 (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal calf serum, 100 units per ml penicillin and 100 units per ml streptomycin in a humidified incubator with 5% CO₂ at 37 °C. Human normal mammary epithelial cell line MCF-10A was maintained in Dulbecco's modified Eagle's medium/F12 (1:1) medium (Life Technologies) supplemented with 15% fetal calf serum, 10 µg ml⁻¹ insulin, 20 ng ml⁻¹ epidermal growth factor, 100 units per ml penicillin and 100 units per ml streptomycin in a humidified incubator with 5% CO₂ at 37 °C. For hypoxic cell culture, cells were incubated with medium containing a hypoxia-mimicking agent, CoCl₂.

Table 1. Primer sequences

Gene	Sequence
5HRE-CEAp	5'-GCG AGA TCT ATT ATG CTA GTC CAC-3' 5'-GCG AAG CTT AGC TTG AGT TCC AGG AAC G-3'
CEAp	5'-GCG AGA TCT CCC GGG ACC CTG CTG-3' GGT TT-3' 5'-GCG AAG CTT AGC TTG AGT TCC AGG AAC G-3'
RASSF1A	5'-GCG AAG CTT ATG TCG GGG GAG CCT GAG CT-3' 5'-ATG GGG TAC CGT CCC AAG GGG GCA GGC GT-3'
CEA	5'-AGT CTA TGC AGA GCC A CC CAA A-3' 5'-CCA CAC TCA TAG GGT CCT ACA TCA-3'
RASSF1A	5'-AGT GCG CGCA TTG CAA GTT-3' 5'-AAG GTC AGG TGT CTC CCA CT-3'
β-Actin	5'-GTG CGT GAC A TT AAG GAG AA-3' 5'-GGA AGG AAG GCT GGA AGA-3'

Amplification and generation of target DNA fragments

RASSF1A cDNA fragment (NM_007182), 5HRE and CEAp were amplified from the pcDNA-RASSF1A (GeneChem Co., Ltd, Shanghai, China) and pLEGFP-N1-5HRE-CEAp-TSST-1-linker-CD80TM,³⁴ respectively, using PCR with the specific primers listed in Table 1. All primers were synthesized by Sangon Biotech (Shanghai, China). The 5HRE-CEAp products were digested with *BglII/HindIII*, whereas the RASSF1A products were digested with *HindIII/KpnI*. The 5HRE-CEAp-RASSF1A (5HC- RASSF1A) fragment was ligated using T4 DNA ligase to link the 5HRE-CEAp and RASSF1A fragments containing *HindIII/KpnI* sites. However, negative control vector just contains all 5HRE-CEAp promoter sequences without RASSF1A cDNA. All sequences were verified by DNA sequence analysis (BGI-Tech, Shenzhen, China).

Lentivirus infection of SGC7901 and MKN28 cells

A lentiviral vector carrying RASSF1A, pLV-5HRE-CEAp-RASSF1A or a negative control vector, pLV-5HRE-CEAp-NC, and corresponding viruses (1×10^8 pfu) were custom constructed and prepared by GeneChem Co., Ltd. Lentivirus infection was performed in the presence of polybrene (GeneChem Co., Ltd.) according to the manufacturer's protocol. Specifically, cells were seeded in 24-well plates overnight at a density of 5×10^4 cells per well and then infected with pLV-5HRE-CEAp-RASSF1A or pLV-5HRE-CEAp-NC lentivirus at a multiplicity of infection of 10 pfu per cell. Three days later, cells were further cultured in a medium containing $2 \mu\text{g ml}^{-1}$ puromycin for SGC7901 and $10 \mu\text{g ml}^{-1}$ for MKN28 for 2 weeks. The drug-resistant clones were used in the following experiments.

Quantitative reverse-transcription PCR

Stably transfected SGC7901/5HC-RASSF1A, SGC7901/NC or MKN28/5HC-RASSF1A cells were subjected to total cellular RNA isolation using an RNA fast 200 kit (Pioneer Biotech, Xi'an, China) according to the manufacturer's instructions. The resulting RNA was reverse transcribed into cDNA using PrimeScript RT Master Mix (Takara, Dalian, China), according to the manufacturer's instructions, and then subjected to quantitative reverse-transcription PCR analysis of RASSF1A mRNA using SYBR Premix Ex Taq II (Takara) and specific primers (Table 1). Each reaction was performed in triplicate and the mean RASSF1A mRNA level in each cell line was calculated by the $2^{-\Delta\Delta\text{CT}}$ method.

Immunocytochemistry

Cells were cultured in 24-well plates with 1×10^4 cells per well for 48 h and then washed with phosphate buffered saline and fixed with 4% paraformaldehyde for 30 min. CEA expression was detected by using a CEA polyclonal antibody (1:100, Proteintech, Chicago, IL, USA) and SP assay and DAB detection kits (ZSGB-BIO, Beijing, China). The negative control used an equal volume of phosphate buffered saline to replace the primary antibody. CEA immunostaining quantification was performed using digital image analysis with the Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA) according to the methods described in a previous study.³⁵ Briefly, images were acquired by a Nikon ECLIPSE Ti-S microscope mounted with a Nikon digital camera (Nikon, Tokyo, Japan). Five randomly selected fields ($\times 400$) were acquired per well. The integrated optical density of the positive field and area of interest was measured. The mean

density (optical density/area of interest) represented the quantification of the specific protein per unit area.

Protein extraction and western blotting

Cells were grown and treated with or without CoCl_2 before homogenization in RIPA buffer containing a protease inhibitor cocktail (Roche, Mannheim, Germany). Cell lysates containing 30 μg total protein were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The membranes were blotted with anti-HIF-1 α , caspase 3, MMP-2, cyclinD1, β -actin monoclonal antibody (Epitomics, Burlingame, USA) or an anti-RASSF1A monoclonal primary antibody (eBioscience, San Diego, USA) at a dilution of 1:1000, followed by goat anti-Rabbit horseradish peroxidase secondary antibody (ABGENT, San Diego, USA). β -Actin was probed with an anti- β -actin monoclonal antibody conjugated with horseradish peroxidase (San Ying Biotechnology, Wuhan, China). Protein bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL, USA).

Luciferase activity assay

To assess pGL4.20-5HRE-CEAp-Luc activity under both normoxic and hypoxic conditions, we constructed reporter vectors based on the vector pGL4.74 (hRluc/TK) and used the Dual-Glo Luciferase Assay System E2920 (Promega, Madison, WI, USA). The promoterless firefly luciferase reporter gene vector pGL4.20 was used as a negative control. The pGL4.74 (hRluc/TK) vector containing the hRluc (*Renilla reniformis*) luciferase reporter gene and a herpes simplex virus thymidine kinase promoter were used as internal controls. 5HRE-CEAp was inserted into multiple cloning sites of the pGL4.20 vector, resulting in the pGL4.20-5HRE-CEAp-Luc recombinant plasmid. For the luciferase activity assay, log-phase growing cells were cultured in 96-well plates with 5×10^3 cells per well. When the cells reached the optimal 70%–90% confluency, the cells were transfected with 200 ng of pGL4.20 or pGL4.20-5HRE-CEAp-Luc plus pGL4.74 (30 ng) using TurboFect Transfection Reagent (Thermo Scientific, Vilnius, Lithuania) according to the manufacturer's protocols. Twenty-four hours after transfection, cells were treated with or without the CoCl_2 hypoxia-mimicking agent for an additional 24 h and collected for the dual luciferase assay using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's protocols. Luminescence was measured using a PerkinElmer EnSpire Multilabel Reader 2300 (PerkinElmer Inc., Waltham, MA, USA). Firefly luciferase activity was normalized to Renilla luciferase activity. For easy comparison, fold changes were used and the data were summarized using the following formulation: sample (Firefly luciferase activity/Renilla luciferase activity)/control (Firefly luciferase activity/Renilla luciferase activity).

Cell viability assay

A cell counting kit (CCK-8, Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to assay cell viability. Briefly, 2000 viable and stably gene-transfected cells per well were seeded into 96-well tissue culture plates to a final volume of 200 μl of the growth medium. The hypoxia group was treated with CoCl_2 after 24 h. The concentration of CoCl_2 was optimized by CCK-8 assay, for example, $50 \mu\text{M l}^{-1}$ for SGC7901 and $100 \mu\text{M l}^{-1}$ for MKN28. After treatment and incubation, each plate was subjected to the CCK-8 assay according to manufacturer's protocols. After 2 h incubation at 37 °C, absorbance at 450 nm was measured with PerkinElmer EnSpire Multilabel Reader 2300 (PerkinElmer Inc.). The data were plotted in a graph (Figure 3) and summarized as the percent of growth inhibition.

Flow cytometric apoptosis and cell cycle distribution assays

SGC7901, SGC7901/5HRE-CEAp-RASSF1A and SGC7901/NC cells were seeded into 60-mm culture dishes and the hypoxia group was treated with CoCl_2 ($300 \mu\text{M l}^{-1}$) for 12 h. For apoptotic analysis, a FITC Annexin V Apoptosis Detection Kit I (Becton Dickinson, Franklin Lakes, NJ, USA) was used to analyze apoptosis according to the manufacturer's instructions. For cell cycle analysis, duplicate cells were fixed overnight with 75% ethanol at -20°C , incubated with RNase A at 37 °C for 30 min and then incubated with propidium iodide at room temperature for 30 min. Cells were examined by flow cytometry and the data were analyzed by CellQuest version 3.3 software (Becton Dickinson).

Colony formation assay

SGC7901, SGC7901/5HRE-CEAp-RASSF1A and SGC7901/NC cells were seeded in 6-well plates at a density of 200 cells per well. All wells were cultured for 2 weeks in selection medium containing $1 \mu\text{g ml}^{-1}$ puromycin. The hypoxic group was also treated with CoCl_2 ($50 \mu\text{M ml}^{-1}$) and, after treatment and incubation, cells were fixed and stained with 1% crystal violet and the cell colonies were counted under an inverted microscope.

Tumor cell invasion assay

A Transwell system was used to assess tumor cell invasion capacity. In brief, upper chamber filters were coated with 50 μl BD Matrigel (Becton Dickinson) according to the manufacturer's instructions; serum-starved cells were trypsinized and plated at 5×10^4 cells per upper chamber of a 24-well plate (8 μm , Corning, New York, NY, USA) and then the lower chambers were filled with 10% fetal bovine serum. For the hypoxia group, CoCl_2 ($300 \mu\text{M l}^{-1}$) was also added to the upper chamber. The cells were allowed to incubate 30 h at 37 °C. After incubation, the non-invasive cells were removed from the upper chamber surface with a cotton swab and the cells under the lower surface were fixed with 4% paraformaldehyde for 20 min and stained with 1% crystal violet solution. Cell invasion was quantified by counting the number of cells in five fields per well under a microscopy.

Nude mouse xenograft assay

SGC7901 cells were subcutaneously injected into the right flank of 6-week-old BALB/c nude mice at a density of 3×10^6 in 100 μl phosphate buffered saline. Tumor volume was calculated using the empirical formula $V = 1/2 \times (\text{the shortest diameter})^2 \times (\text{the longest diameter})$. After xenograft tumors had reached 50–100 mm^3 , mice were randomly divided into three groups: negative solution, LV-NC and LV-5HRE-CEAp-RASSF1A (LV-5HC-RASSF1A). Lentiviruses (1×10^8 pfu) in 100 μl serum-free medium were administered intratumorally at three sites per tumor. Three days later, lentivirus administration was repeated. In the negative solution group, 100 μl serum-free RPMI1640 was injected twice instead of the recombinant virus. Tumor size and growth were monitored and measured using a caliper at regular intervals. Mice were killed 2 weeks after the second virus injection and xenograft tumors were collected, measured and photographed. Xenograft tumors were fixed in 10% buffered formalin and embedded in paraffin for sectioning and immunohistochemical staining of RASSF1A expression.

Statistical analysis

All data are representative of three independent experiments and are presented as the mean \pm s.d. A two-sample *t*-test was performed to analyze two independent samples, whereas analysis of variance was conducted for comparison among groups. SPSS 13.0 software (SPSS, Chicago, IL, USA) was used to calculate the *P*-value and a *P* < 0.05 was considered to be statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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