

Mesenchymal stem cell-based *NK4* gene therapy in nude mice bearing gastric cancer xenografts

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Abstract: Mesenchymal stem cells (MSCs) have been recognized as promising delivery vehicles for gene therapy of tumors. Gastric cancer is the third leading cause of worldwide cancer mortality, and novel treatment modalities are urgently needed. *NK4* is an antagonist of hepatocyte growth factor receptors (Met) which are often aberrantly activated in gastric cancer and thus represent a useful candidate for targeted therapies. This study investigated MSC-delivered *NK4* gene therapy in nude mice bearing gastric cancer xenografts. MSCs were transduced with lentiviral vectors carrying *NK4* complementary DNA or enhanced green fluorescent protein (GFP). Such transduction did not change the phenotype of MSCs. Gastric cancer xenografts were established in BALB/C nude mice, and the mice were treated with phosphate-buffered saline (PBS), MSCs-GFP, Lenti-*NK4*, or MSCs-*NK4*. The tropism of MSCs toward gastric cancer cells was determined by an in vitro migration assay using MKN45 cells, GES-1 cells and human fibroblasts and their presence in tumor xenografts. Tumor growth, tumor cell apoptosis and intratumoral microvessel density of tumor tissue were measured in nude mice bearing gastric cancer xenografts treated with PBS, MSCs-GFP, Lenti-*NK4*, or MSCs-*NK4* via tail vein injection. The results showed that MSCs migrated preferably to gastric cancer cells in vitro. Systemic MSCs-*NK4* injection significantly suppressed the growth of gastric cancer xenografts. MSCs-*NK4* migrated and accumulated in tumor tissues after systemic injection. The microvessel density of tumor xenografts was decreased, and tumor cellular apoptosis was significantly induced in the mice treated with MSCs-*NK4* compared to control mice. These findings demonstrate that MSC-based *NK4* gene therapy can obviously inhibit the growth of gastric cancer xenografts, and MSCs are a better vehicle for *NK4* gene therapy than lentiviral vectors. Further studies are warranted to explore the efficacy and safety of the MSC-based *NK4* gene therapy in animals and cancer patients.

Keywords: gastric cancer, gene therapy, tumor xenograft, hepatocyte growth factor, lentivirus, angiogenesis, apoptosis

Introduction

Stomach or gastric cancer is the fifth most common cancer and the third leading cause of death from cancer globally with approximately 952,000 new cases and 723,000 deaths making up 7% of all cancer cases and 9% of deaths.^{1,2} Almost two-thirds of gastric cancer cases occur in developing countries and 42% in the People's Republic of China accounting for 3.99% of all deaths.³ The Republic of Korea had the highest rate of stomach cancer, followed by Mongolia, Japan, Guatemala, People's Republic of China, Tajikistan, Kazakhstan, Kyrgyzstan, Albania, and Belarus. There are about 22,220 new cases of stomach cancer and 10,990 deaths every year in the United States. In the United Kingdom, 7,089 people were diagnosed with stomach cancer and 4,830 deaths due to gastric cancer were recorded in 2011.³ Primary treatment modalities for stomach cancer include surgery, chemotherapy, radiation therapy, and biological therapy. Despite advances in past decades in treatment of early gastric cancer, early-stage disease accounts

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for only 10% to 20% of all cases diagnosed and most gastric cancer patients are still diagnosed with advanced stage disease and their 5 years relative survival rate is as low as <10%.^{2–5} Therefore, it is necessary to identify novel therapeutic strategies for later stage gastric cancer. Gene therapies for solid tumors have been extensively investigated in animal models, but in clinical trials, very few gene therapies have demonstrated antitumor effects on advanced solid tumours.⁶ This poor outcome relates, at least in part, to our inability to deliver therapeutic agents specifically and efficiently to the tumor tissues.⁷ Selective gene transfer to the target organ/cells, eg, the stomach cancer, is a major challenge for gene therapy.⁸

Towards this end, our research is focused on mesenchymal stem cells (MSCs) as a gene delivery vehicle. These plastic, adherent cells isolated from bone marrow are capable of self-renewal and have the potential to differentiate into mesenchymal and non-mesenchymal tissues.^{9,10} Thus, they may be advantageous for tumor gene therapy because MSCs exhibit tropism to the sites of tissue damage as well as cancer lesions.^{11,12} Migration of MSCs to tumor lesions is thought to be due to similarities in the inflammatory milieu produced by healing wounds and tumors, evoking the notion that “tumors are wounds that never heal”.¹³ To date, MSCs have been investigated in the treatment of a number of diseases, such as myocardial infarction, Alzheimer’s disease, Parkinson’s disease, Crohn’s disease, diabetes, stroke, pulmonary arterial hypertension, and cancer.^{14–17} Furthermore, MSCs can be used for autologous transplantation to avoid host immune response.^{14,18,19} The homing capacity of MSCs has been demonstrated with almost all tested human cancer cell lines.^{20–22}

Hepatocyte growth factor (HGF) was first identified and cloned as a mitogenic polypeptide for hepatocytes.²³ Subsequent studies have revealed that HGF is a multifunctional growth factor that stimulates mitogenesis and morphogenesis in a variety of epithelial and endothelial cells.²⁴ The receptor for HGF is the c-Met proto-oncogene product.²⁵ HGF also mediates tumor–stromal interactions that confer invasive, angiogenic, and metastatic potentials to cancer through HGF/c-Met receptor binding.^{26–29} NK4 is composed of the N-terminal hairpin and subsequent four kringle domains of HGF.^{30–32} NK4 not only acts as a competitive antagonist for HGF but also independently acts as a potent angiogenesis inhibitor.^{30,33,34} Considering these bifunctional properties (angiogenesis inhibitor and HGF antagonist), NK4 gene therapy could potentially be an ideal treatment for human cancer.^{32,35} For example, virus vector-mediated NK4 gene therapy suppressed tumor growth and metastasis in nude mice.^{36–38} However, the systemic administration of viral vectors often causes severe adverse

effects such as severe liver toxicity and immune responses.³⁹ In this regard, we investigated MSCs as a gene delivery vehicle carrying NK4 gene therapy to gastric cancer xenografts in nude mice in comparison to a lentiviral vector carrying NK4 and other controls in this study.

Materials and methods

Culture and identification of human MSCs

Human MSCs were isolated by adherence purification as described previously by Mohr et al⁴⁰ from the bone marrow of young patients with femur fracture during open reduction and internal fixation in our hospital. These patients were otherwise healthy before hospitalization. The separated MSCs were subcultured at a density of 6,000–8,000 cell/cm² in a low-glucose Dulbecco’s Modified Eagle’s Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (HyClone, Thermo Fisher Scientific), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Thermo Fisher Scientific) and used for experiments during passages 3 to 5. The cell markers CD34, CD44, CD45, and CD105 were used to identify the MSCs upon fluorescence-activated cell sorter (FACS, Becton, Dickinson and Company, NJ, USA) with specific antibodies (eBioscience, San Diego, CA, USA). This study was approved by the Ethics Committee of the First Affiliated Hospital, Nanchang University, Nanchang, Jiangxi, People’s Republic of China, and each patient signed an informed consent form.

Cell lines and culture

A human gastric carcinoma cell line MKN45 was kindly provided by Dr Dai-Ming Fan of Xi-Jing Hospital of Digestive Diseases, Xi’an, Shanxi, People’s Republic of China, and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific). Human fibroblasts were kindly provided by Dr Li-Xin Liao of the Department of Burn Surgery, The First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi, People’s Republic of China, and cultured in low-glucose DMEM. A normal human gastric epithelial cell line, GES-1, was cultured in high glucose DMEM. All media were supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂/95% air.

NK4 lentiviral vector construction, production, and cell transduction

The *NK4* gene was acquired by polymerase chain reaction from human *HGF* complementary DNA (cDNA) (German

Resource Center RZPD, Berlin, Germany). The primers used to amplify A fragment (521 bp) of *NK4* cDNA gene from human *HGF* cDNA included: forward, 5'-GAGGATCCCCGGGTACCGGTGCCACCATGTGGGTGACCAAACTCC-3', and reverse, 5'-CGAAGGCAAAAAGCTGTGTTCGTGTGGTATCATGG-3'; and the primers to amplify B fragment (976 bp) of *NK4* cDNA gene from *HGF* cDNA: forward, 5'-CACAGCTTTTTGCCTTCGAGCTATCGGGGTAAAGACC-3', reverse, 5'-TCACCATGGTGGCGACCGGGACTATTGTAGGTGTGGTATC-3'. A and B fragments were used as the templates for amplification of full-length *NK4* cDNA gene (1,479 bp), and the primers were: forward, 5'-GAGGATCCCCGGGTACCGGCGCCACCATGTGGGTGACCAAACTCC-3', and reverse, 5'-TCACCATGGTGGCGACCGGGACTATTGTAGGTGTGGTATC-3'. A lentiviral plasmid pGC-FU carrying the enhanced green fluorescent protein (GFP) gene (GeneChem Co., Ltd., Shanghai, People's Republic of China) was used as a backbone for subcloning the *NK4* fragment. Purified polymerase chain reaction products containing the *NK4* gene coding sequence were ligated with pGC-FU-GFP vector by In-Fusio convertase (BD Biosciences, San Jose, CA, USA). The recombinant pGC-FU-GFP-NK4-plasmids, the construction plasmids Helper1.0, and the envelope plasmids Helper2.0 (GeneChem Co., Ltd.) were cotransfected into human embryonic kidney epithelial 293T cells mediated by Lipofectamine 2000 (Thermo Fisher Scientific). Lentiviral vectors carrying the *NK4* fragment (Lenti-NK4) or GFP (Lenti-GFP) were produced and the lentiviral titer was detected as described previously.⁴¹

After production of these lentiviral vectors, MSCs were transduced with Lenti-NK4 (MSCs-NK4) or Lenti-GFP (MSCs-GFP) at a multiplicity of infection (MOI) of 50. NK4 constructed in the lentiviral plasmid was in a secreting form and NK4 was continuously expressed and released from MSCs. Expression of NK4 was detected by enzyme linked immunosorbent assay (ELISA) and Western blotting assay as previously described.³⁹ GFP expression was analyzed by flow cytometry using the FACSCalibur system (Becton Dickinson Co., Franklin Lakes, NJ, USA) or a fluorescence microscope (LSM700, Carl Zeiss Meditec AG, Jena, Germany).

Protein extraction and Western blotting analysis

MSCs-NK4, MSCs-GFP, or MSCs were lysed in a buffer containing 0.5% Lubrol-PX, 50 mM KCl, 2 mM CaCl₂, 20% glycerol, 50 mM Tris-HCl at pH 7.4, 0.1% protease, and 1% phosphatase inhibitors (Sigma-Aldrich Co., St Louis,

MO, USA), and all cellular lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories Inc., Hercules, CA, USA). After incubation with a rabbit polyclonal anti-HGF antibody at a dilution of 1:1,000 (Santa Cruz Biotechnology Inc., Dallas, TX, USA), the blots were washed with phosphate-buffered saline (PBS) and then incubated with a goat anti-rabbit Immunoglobulin G conjugated with horseradish peroxidase (Santa Cruz Biotechnology Inc.). Bands were visualized by enhanced chemiluminescence (Thermo Fisher Scientific). Recombinant human HGF (Peprotech, Rocky Hill, NJ, USA) was used as a positive control.

Cell migration assay

The tropism of MSCs to gastric cancer cells was determined using a Transwell migration assay with 8 μm pore size membranes (Corning Incorporated, Corning, NY, USA). MNK45 or GES-1 cells were cultured for 24 hours in serum-free medium and then plated into the bottom wells. MSCs, MSCs-NK4, or human fibroblasts (2×10⁴) in 300 μL serum-free medium were seeded into the upper chamber and cultured for 24 hours at 37°C. The MSCs, MSCs-NK4, or human fibroblasts that had attached to the top side of the membrane were removed by a cotton swab, and the cells that had migrated onto the bottom side were fixed with 4% paraformaldehyde, stained using crystal violet, and counted (five fields per well for triplicate wells) at 10× magnification with an Olympus BH-2 microscope (Olympus Corporation, Tokyo, Japan).

Nude mouse xenograft cancer model

BALB/C nude mice (6–8 weeks old) from the National Institutes for Food and Drug Control Laboratory Animal Center (Beijing, People's Republic of China) were bred and kept under standard pathogen-free conditions. All animal studies were performed in accordance with institutional guidelines under the approved protocols. Xenografts were initiated by subcutaneous injection of 2×10⁷ MKN45 cells in 200 μL PBS into the right flank near the axillary fossa. The mouse was euthanized when the longest diameter of the tumor reached 10 mm, and the tumor was aseptically dissected and mechanically minced. Then 2 mm³ pieces of tumor tissue were transplanted into the right flank near the axillary fossa of mice. Tumors were measured every 3 days with calipers. When tumors were 1.5 cm in the longest diameter, mice were randomly assigned to one of four experimental groups.

Treatment of gastric cancer nude mouse xenografts

To evaluate the therapeutic effects of MSCs-NK4 *in vivo*, tumor-bearing nude mice were injected with MSCs-GFP or MSCs-NK4 (6×10^5 cells in 0.2 mL PBS), Lenti-NK4 (3×10^7 viral particles), or PBS (0.2 mL) in the lateral tail vein for systemic administration at 1, 7, 14, and 21 days (eight animals per group). Three different diameters of each tumor were measured twice a week. The tumor volume was calculated using the formula: $\pi/6 (0.5326) \times \text{length (mm)} \times \text{width (mm)} \times \text{height (mm)}$.⁴² Animals were sacrificed on the 28th day after injections, and none of the mice had to be sacrificed during experiment due to tumor ulceration, bleeding, or moribund state with excessive weight loss exceeding 25% of initial weight before the experiment. Tumor xenografts were excised and weighed to assess tumor burden. Results were evaluated as mean tumor volume or weight \pm standard error (SEM).

Histopathological examination of tumor xenografts

Tumor xenografts were fixed in 4% buffered formalin and embedded in paraffin for sectioning and hematoxylin and eosin (H & E) staining. Histopathology was evaluated blindly under a light microscope by a pathologist. Tumor necrosis was scored according to the method described previously.⁴³ In brief, necrotic tissues observed in 1%–20% of the regions of interest were scored as 1, and 21%–40%, 41%–60%, 61%–80%, and 81%–100% of necrotic tissues were scored as 2, 3, 4, and 5, respectively. In contrast, if necrosis occupied <1% of the regions of interest, the score was 0. Next, the mean regions of the interest were calculated and designated as the score of the tumor xenograft. Partial tumor xenografts were snap-frozen for fluorescence microscopy observation of MSCs in fresh cryosections (6–8 μm).

Immunohistochemistry

Paraffin-embedded tumor sections (4 μm) were used for immunostaining of proliferating cell nuclear antigen (PCNA) with a monoclonal primary mouse anti-human PCNA antibody at a dilution of 1:100 (from Santa Cruz Biotechnology Inc.). The proliferative index was defined as the percentage of positively stained cells among 100 nuclei from five randomly selected fields at 200 \times magnification. The microvessel density was assessed using a monoclonal mouse anti-human CD31 antibody (1:100; Santa Cruz Biotechnology Inc.). Any distinct area of positive staining for CD31 was counted as a single vessel. The number of vessels was counted in five

fields at 200 \times magnification, and the results were expressed as the mean number of vessels \pm SEM. The negative control sections for PCNA and CD31 were obtained by omitting the primary antibody incubations.

TUNEL apoptosis assay

Cellular apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) using an *in situ* apoptosis detection kit (Promega Corporation, Fitchburg, WI, USA) according to the manufacturer's instructions. For the positive control, the section was treated with DNase I, whereas the negative control was prepared with the omission of terminal deoxynucleotidyl transferase incubation. The total number of apoptotic cells in five randomly selected fields (400 \times magnification) per tumor xenograft was counted under a microscope. The apoptotic index was expressed as the percentage of positively stained cells among 100 nuclei per field.

Statistical analysis

Data are presented as the mean \pm SEM. One-way analysis of variance (ANOVA) was used to compare the means from multiple experimental groups, followed by Dunnett's post hoc test. Statistical differences were considered to be significant when $P < 0.05$ in a two-tailed test.

Results

Isolation and culture of human MSCs

Attached cells were observed on day three after the initial plating of human MSCs. Our data showed that the cultures underwent an initial lag phase growth lasting approximately 6 days, during which the colonies were seen to arise from single cells. Cells had a typical spindle shape, consistent with the morphology reported by others (Figure 1A and B).^{44,45} Cells were primarily expanded when they reached confluence after 10–14 days' culture. A population of fibroblast-like MSCs were observed microscopically after cells were expanded in three passages (Figure 1C) and verified using flow cytometry. The isolated cells were negative for typical hematopoietic antigens CD45 and CD34 but were positive for CD44 and CD105 in each culture (Figure 1D). Therefore, the isolated and cultured cells exhibited typical properties of human MSCs as described previously.^{44–49}

Transduction efficiency of MSCs with lentiviral vectors and expression of NK4 in Lenti-NK4-transduced MSCs

Next, MSCs were stably transduced with Lenti-NK4 or Lenti-GFP, optimized using different MOIs (10–100). The data

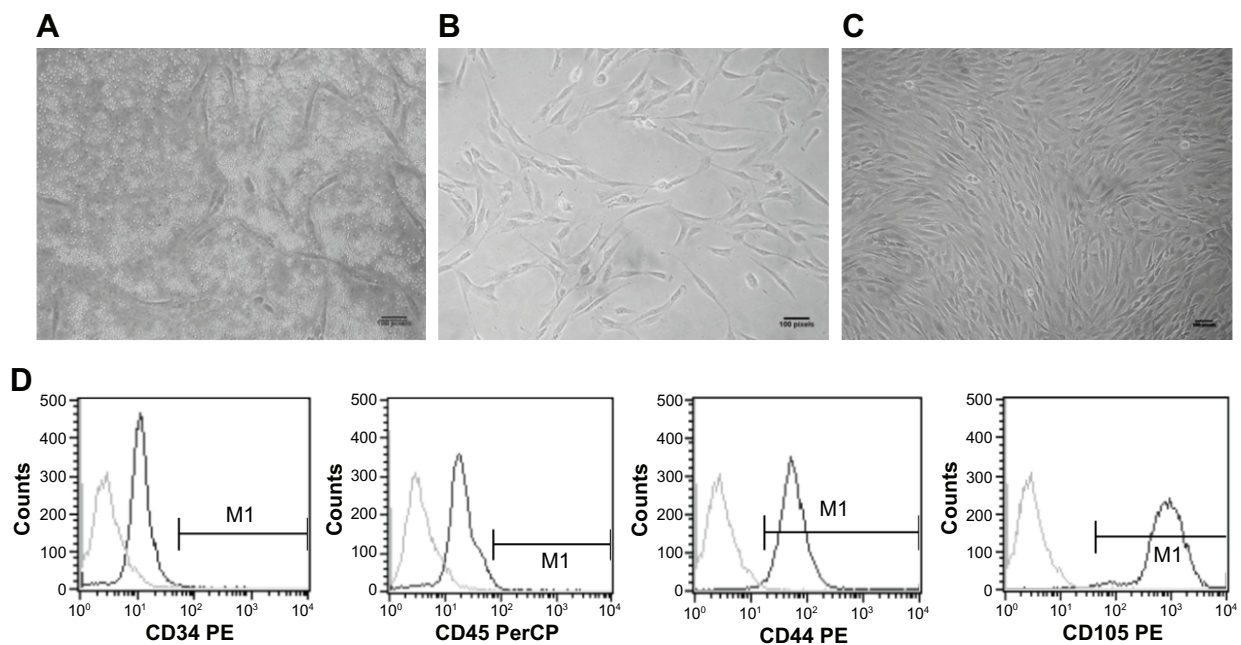


Figure 1 Isolation, culture and identification of human mesenchymal stem cells (MSCs).

Notes: MSCs were collected from the bone marrow of young patients with femur fracture. **(A)** MSCs on the third day of primary culture, **(B)** MSCs on the seventh day of primary culture, **(C)** MSCs at passage 3, and **(D)** characterization of MSCs phenotype using flow cytometry. Human MSCs isolated were found to be negative for CD34 and CD45 but positive for CD44 and CD105.

Abbreviations: PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

showed that GFP expression in the MSCs was increased with increasing MOI. MSCs tolerated an MOI of 50, and this MOI was used for further experiments, producing a more than 85% successful transduction rate at day three with 4.26% cell death only (Figure 2A and B). Moreover, the NK4 level in cell culture supernatants was also significantly increased with increasing MOI up to 100 ($P < 0.001$, by one-way ANOVA; Figure 2C). At an MOI of 50, the NK4 level in the cell culture supernatants of MSCs-NK4 was increased and reached the highest level at day five (2473.38 ± 155.82 pg/mL, Figure 2D). GFP expression in MSCs-NK4 was also increased over 3 days of incubation (Figure 2E). The Western blot data showed that the NK4 protein was also present in Lenti-NK4-infected MSC lysates, but not in that of Lenti-GFP or non-infected MSCs (Figure 2F). The phenotype of the MSCs-NK4 and MSCs-GFP was the same as the non-transduced MSCs, suggesting that the lentiviral-mediated transduction did not alter the phenotypes of MSCs (Figure 3).

Tropism of MSCs toward gastric cancer cells in vitro

It has been reported that factors released from cancer cells may be potential chemoattractants for MSC tropism.¹¹ To test the ability of the isolated MSCs to migrate toward gastric cancer cells, an in vitro cell migration assay was performed. Transwell cell culture with MKN45 cells to attract MSCs was

used, whereas an immortalized human gastric epithelial cell line GES-1 and the medium alone were used as controls. The data showed that migration of MSCs-NK4 was significantly stimulated by the medium conditioned with MKN45 cells compared to GES-1-conditioned medium or medium alone ($P < 0.001$, by one-way ANOVA; Figure 4A and B). We also confirmed that non-infected MSCs significantly migrated toward MKN45 cells compared to GES-1 cells or medium alone ($P < 0.01$; Figure 4A and B). These data indicate that human gastric cancer cells are capable of stimulating the migration of MSCs and the migratory ability of MSCs was not affected by lentiviral transduction. Medium conditioned by MKN45 cells significantly stimulated the migration of MSCs and MSCs-NK4 but not human fibroblasts. There was no significant increase in the migration of human fibroblasts to MKN45 cells compared to GES-1 cells or medium alone ($P > 0.05$, by one-way ANOVA; Figure 4A and B). These data suggest that MSCs could migrate preferably toward gastric cancer cells, but human fibroblasts do not show this characteristic.

Effects of systemic administration of MSCs-NK4 on tumor growth in nude mice bearing gastric cancer xenografts

To assess the effects of MSCs-NK4 on the treatment of gastric cancer xenografts, we first established gastric cancer

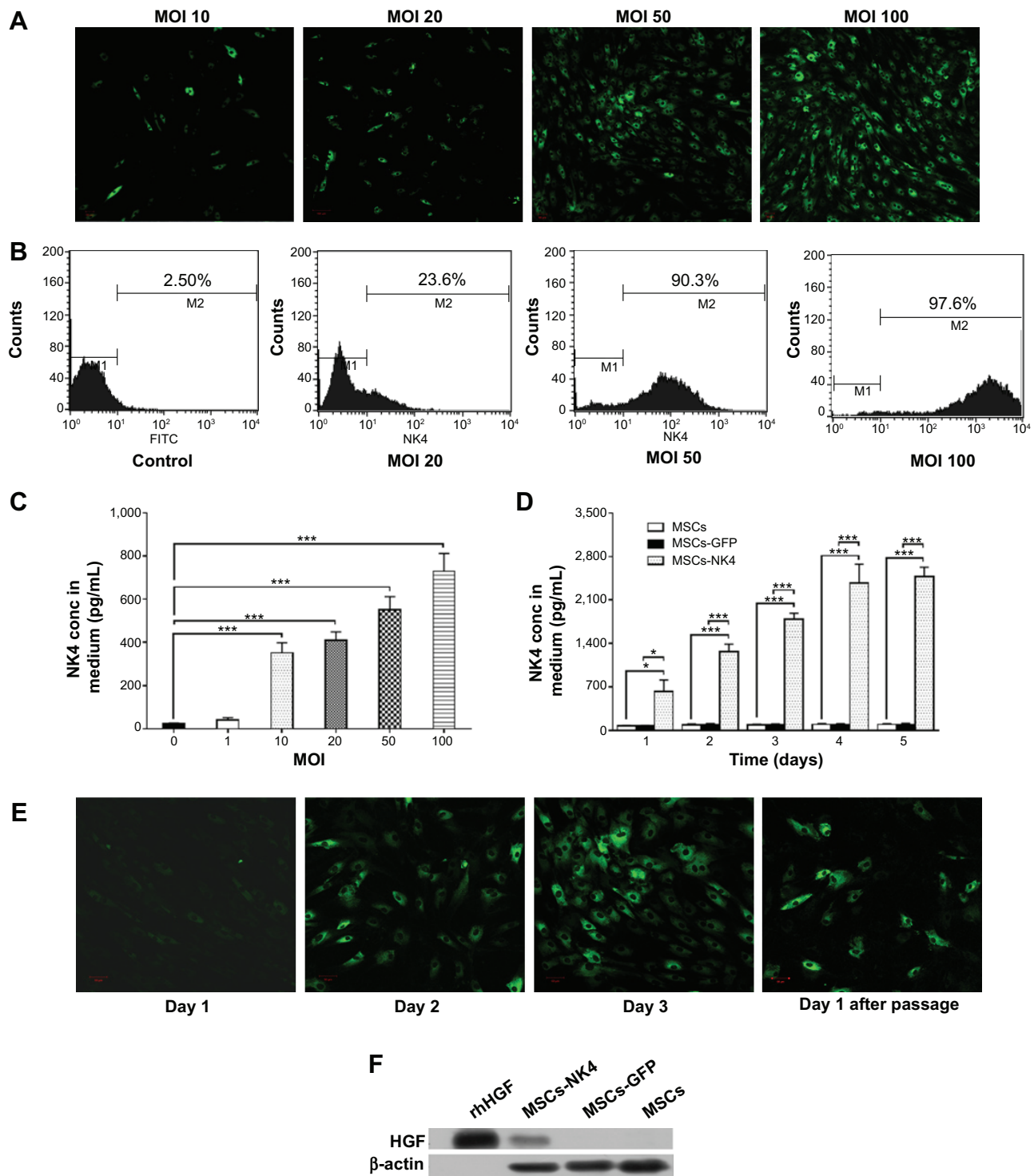


Figure 2 Transduction of NK4 cDNA into human MSCs using a lentiviral vector and characterization.

Notes: The recombinant pGC-FU-GFP-NK4-plasmids, the construction plasmids Helper 1.0, and the envelope plasmids Helper2.0 (GeneChem Co., Ltd., Shanghai, People's Republic of China) were first cotransfected into 293T cells. MSCs were then transduced with Lenti-NK4 (MSCs-NK4) or Lenti-GFP (MSCs-GFP) at varying multiplicity of infection (MOI). (A) Expression of GFP in MSCs observed at day three after Lenti-NK4 infection at MOI of 10, 20, 50, or 100 under fluorescence microscopy, (B) transduction efficiency of Lenti-NK4 in MSCs determined by flow cytometry with an GFP marker which was 87.8% of the enriched GFP-expressing MSC population upon sorting with an MOI of 50, (C) effect of transduction with Lenti-NK4 at different MOIs from 1 to 100 in MSCs on the production of NK4 in the culture medium, (D) effect of time on the production of NK4 in the culture medium after MSCs were transduced with Lenti-NK4 (MOI =50), Lenti-GFP (MOI =50), or not transduced, (E) MSCs-NK4 with increased GFP expression observed at different time points (MOI =50, from Day 1 to Day 3), and (F) Western blot analysis of NK4-GFP fusion protein with a molecular weight of 84 kDa. Recombinant human HGF (Mr =83 kDa) was used as a positive control. * $P < 0.05$; *** $P < 0.001$ MSCs-NK4 versus control groups, by one- or two-way ANOVA.

Abbreviations: GFP, green fluorescent protein; conc, concentration; HGF, hepatocyte growth factor; ANOVA, analysis of variance; rhHGF, recombinant human hepatocyte growth factor; MOI, multiplicity of infection; Mr, molecular weight; MSCs, mesenchymal stem cells; cDNA, complementary DNA.

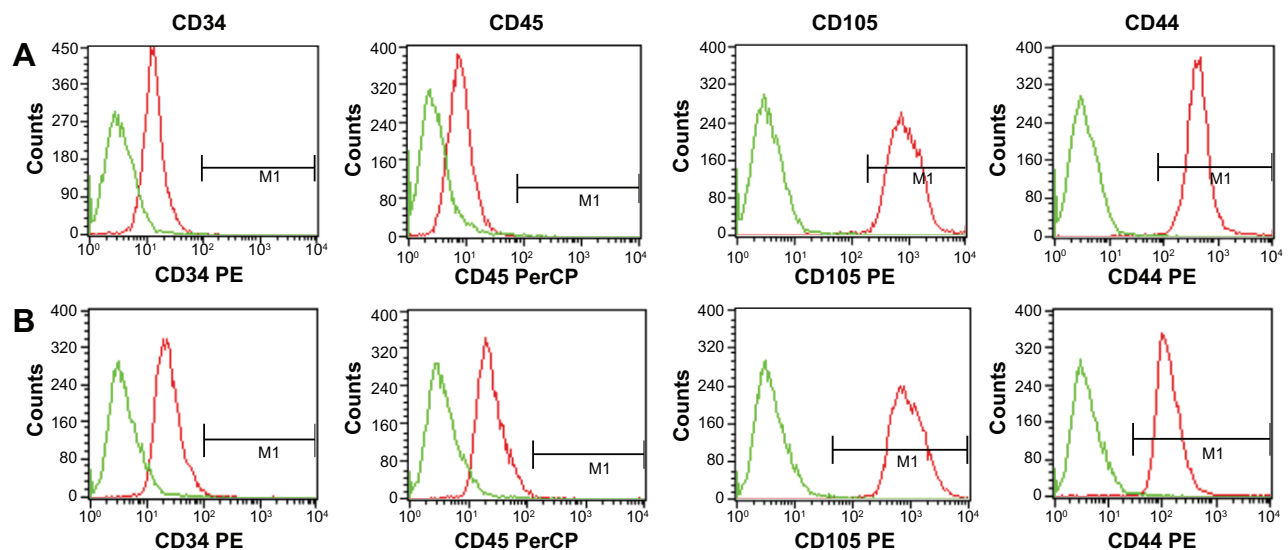


Figure 3 Characterization of MSCs-NK4 (A) and MSCs-GFP (B) phenotypes.

Notes: After transduction of lentiviral vectors (Lenti-NK4 or Lenti-GFP), MSCs-NK4 and MSCs-GFP remained negative for CD45 and CD34 but positive for CD44 and CD105.

Abbreviations: MSCs, mesenchymal stem cells; GFP, green fluorescent protein; PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

cell MKN45 tumor xenografts in nude mice and then systemically administrated MSCs-NK4 into nude mice. Among all treatment groups, MSCs-NK4 produced the smallest tumor volume in nude mice (Figure 5A and B). The average tumor volume in MSCs-NK4 treated mice was significantly decreased compared to that in nude mice treated with PBS or MSCs-GFP at day 21, and this antitumor effect continued through day 28 ($P < 0.05$ or 0.001 , by two-way ANOVA; Figure 5A–C). However, there were no detectable differences in tumor volume between Lenti-NK4 treated mice, PBS injected mice, and MSCs-GFP treated mice at days 7, 14, and 28 ($P > 0.05$ by two-way ANOVA; Figure 5A–C). The tumor mass in MSCs-NK4 treated mice was smaller than that in Lenti-NK4 treated mice ($P < 0.05$ by two-way ANOVA; Figure 5A–C). The tumor growth inhibition rate in MSCs-NK4- and Lenti-NK4 treated mice was 52.2% and 29.4%, respectively at day 28. The tumor weight in MSCs-NK4 treated mice was the lowest among all groups (1.94 ± 0.67 g versus 2.16 ± 0.49 g in Lenti-NK4 treated mice, 2.87 ± 0.86 g in MSCs-GFP treated mice, or 2.84 ± 0.65 g PBS treated mice), although did not achieve statistical significance (Figure 5D). These data demonstrate that NK4-secreting MSCs significantly inhibited the growth of gastric cancer xenografts in nude mice.

Moreover, tumor sections displayed necrosis at different degrees in each group of mice (Figure 5E). The scores of tumor tissue necrosis were highest in the MSCs-NK4 treated mice among all treatment groups of mice, although did not

achieve statistical significance (Figure 5F). These data suggest that MSC-based NK4 gene therapy was able to induce tumor necrosis.

To determine the presence of MSCs-NK4 in tumor xenografts of nude mice, frozen sections of tumor xenografts were analyzed by fluorescence microscopy. Green fluorescence was obviously observed within tumor tissues treated with MSCs-NK4, whereas only faint fluorescent signal was visible in cryosections from Lenti-NK4 treated mice (Figure 5G). These results indicate that MSCs-NK4 migrated and accumulated in tumor tissues after systemic administration to the circulation in vivo. Therefore, MSCs are a better vehicle for NK4 gene therapy than lentiviral vectors.

Inhibition of intratumoral angiogenesis and induction of apoptosis in MSCs-NK4 treated tumor xenografts

The microvessel density of MSCs-NK4 treated tumor xenografts was 5.6 ± 1.47 per microscopic field and less than that of the MSCs-GFP or PBS treated mice (16.4 ± 6.16 and 17.4 ± 9.38 /high power field, respectively), but did not achieve statistical significance probably due to remarkable standard errors (Figure 6A and B). The microvessel density was also decreased in Lenti-NK4 treated tumor xenografts (12.3 ± 2.65 /high power field) compared to MSCs-GFP or PBS treated mice. Furthermore, the apoptotic index, as determined by the percentage of TUNEL-positive cells, was significantly increased in MSCs-NK4 treated tumor

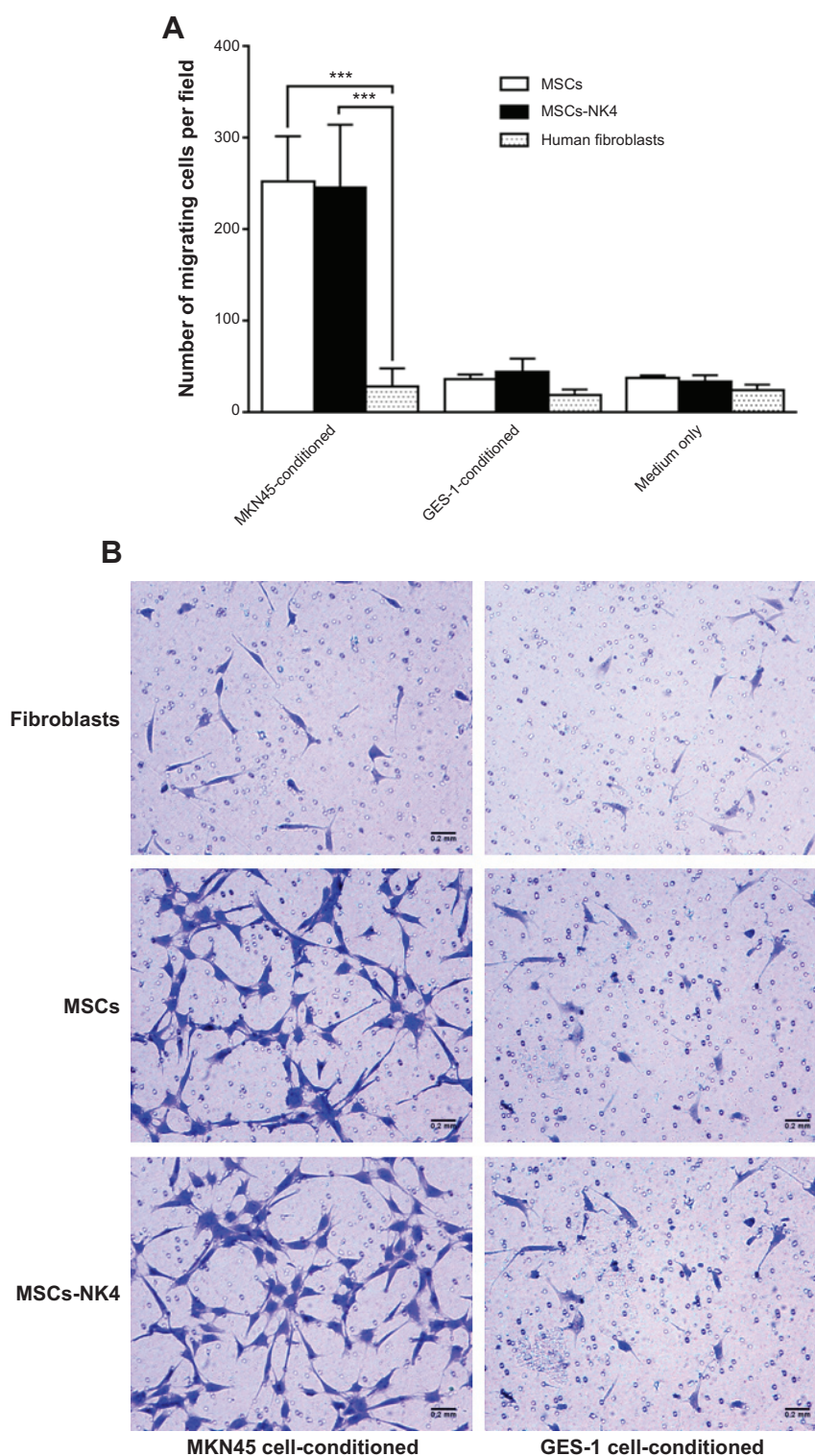


Figure 4 Effects of MKN45 or GES-1 cell-conditioned medium on the migratory ability of MSCs and MSCs-NK4 toward gastric cancer cells determined using a Transwell migration assay.

Notes: MKN45 or GES-1 cells were cultured for 24 hours in serum-free medium and then plated onto the bottom wells. MSCs, MSCs-NK4, or human fibroblasts cultured in serum-free medium were seeded onto the upper chamber and cultured for 24 hours. The MSCs, MSCs-NK4, or human fibroblasts that attached to the top side of the membrane were removed, and the cells that migrated onto the bottom side were fixed, stained, and counted (five fields per well) at 10 \times magnification using a microscope. **(A)** MKN45 cell-conditioned medium significantly stimulated the directional migration of MSCs and MSCs-NK4 compared to human fibroblasts. MSCs and MSCs-NK4 significantly migrated to MKN45 cell-conditioned medium, whereas GES-1 cell-conditioned medium or unconditioned medium did not promote the directional migration of MSCs or MSCs-NK4. $***P < 0.001$, by two-way ANOVA. **(B)** Representative photomicrographs of stained filters showing migrated MSCs and MSCs-NK4 when MKN45 or GES-1 cells were cultured at the bottom side of the well. Transwell migration assay from Corning Incorporated, Corning, NY, USA.

Abbreviations: MSCs, mesenchymal stem cells; ANOVA, analysis of variance.

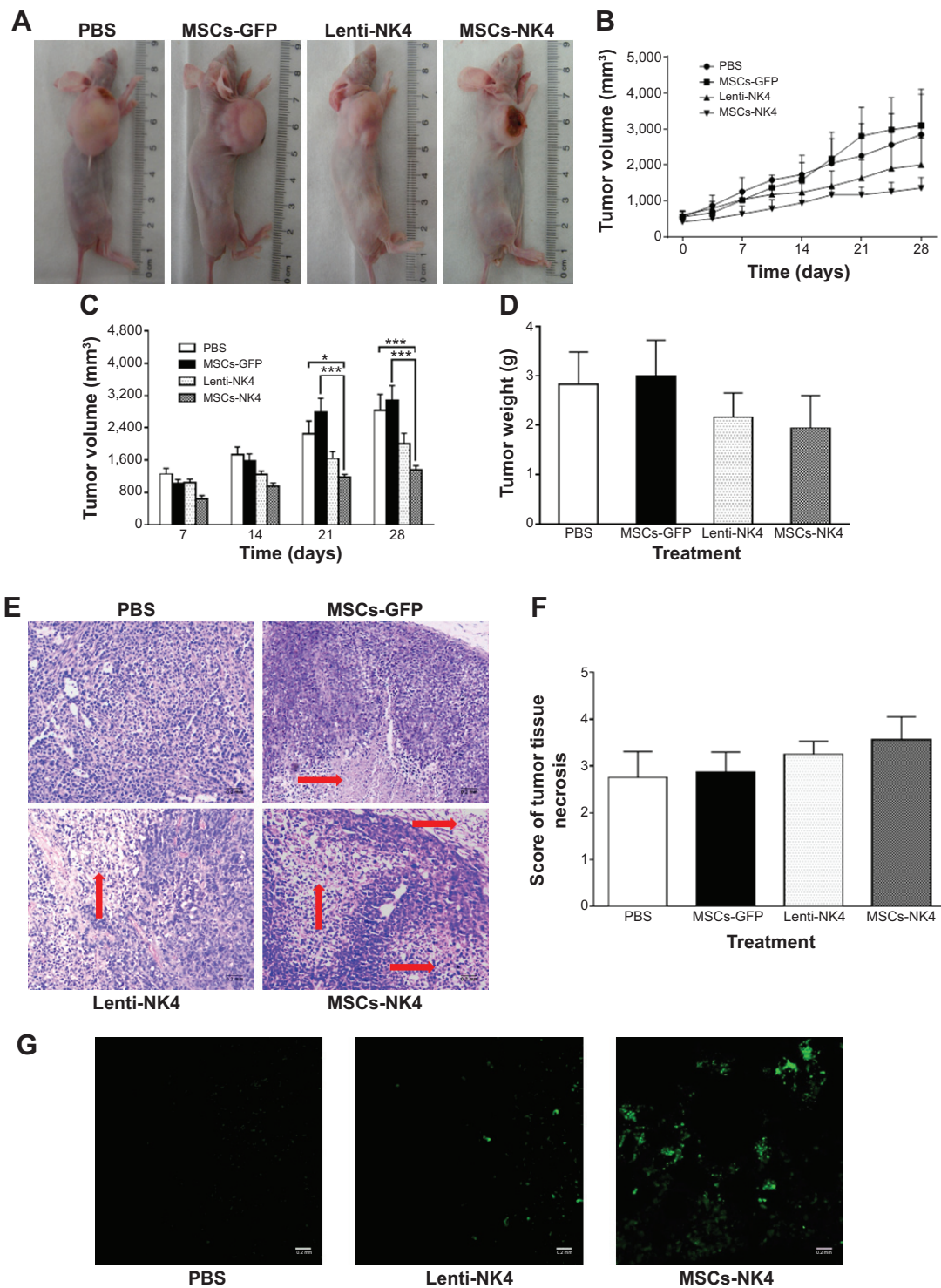


Figure 5 Effects of systemic administration of PBS, MSCs-GFP, Lenti-NK4, or MSCs-NK4 on the growth of gastric tumor xenografts over 28 days in BALB/C nude mice. **Notes:** Tumor-bearing BALB/C nude mice were injected with MSCs-GFP or MSCs-NK4 (6×10^5 cells in 0.2 mL PBS), Lenti-NK4 (3×10^7 viral particles), or PBS (0.2 mL) in the lateral tail vein for systemic administration at 1, 7, 14, and 21 days (eight animals per group). Three different diameters of each tumor were measured twice a week. Animals were sacrificed on the 28th day after injections, tumor xenografts were excised and weighed. **(A)** Representative tumor-bearing mice treated with PBS, MSCs-GFP, Lenti-NK4, or MSCs-NK4, **(B)** the growth curve of tumor xenografts in nude mice. The mice injected with MSCs-NK4 exhibited obvious inhibition of tumor xenograft growth compared to other treatment groups. **(C)** Tumor volume at days 7, 14, 21, and 28 in mice injected with PBS, MSCs-GFP, Lenti-NK4, or MSCs-NK4. The tumor volume in mice treated with MSCs-NK4 was smallest among all groups. **(D)** Tumor weight in nude mice at day 28 after treatment with PBS, MSCs-GFP, Lenti-NK4, or MSCs-NK4. Tumor burden in MSCs-NK4 treated mice was lower than mice treated with PBS or MSCs-GFP. **(E)** Histological score of necrosis in tumor xenografts in nude mice treated with PBS, MSCs-GFP, Lenti-NK4, or MSCs-NK4. The tumor necrosis scores in MSCs-NK4 treated mice were highest among all treatment groups. **(F)** H & E staining of tumor tissues observed by light microscopy where tumor necrosis areas are indicated by arrows, and **(G)** presence of MSCs-NK4 in tumor tissues in vivo after nude mice were injected with MSCs-NK4 via the tail vein. Tumor xenografts were snap-frozen, cryosectioned and then examined under fluorescent microscope. * $P < 0.05$; *** $P < 0.001$, by two-way ANOVA. **Abbreviations:** PBS, phosphate-buffered saline; MSCs, mesenchymal stem cells; GFP, green fluorescent protein; H & E, hematoxylin and eosin; ANOVA, analysis of variance.

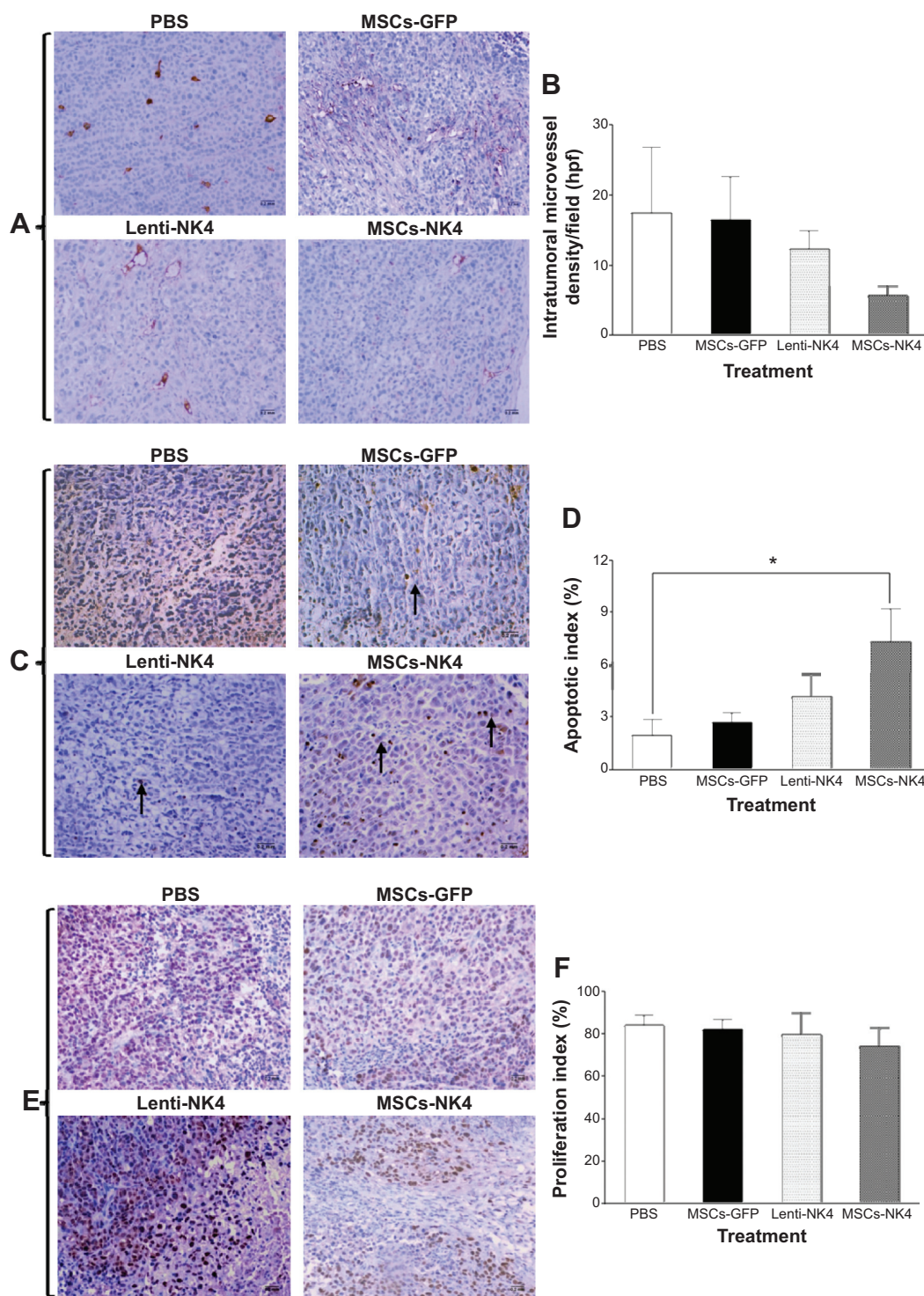


Figure 6 Effects of treatment with PBS, MSCs-GFP, Lenti-NK4, or MSCs-NK4 on intratumoral microvessel density, cellular apoptosis and proliferation in gastric cancer xenografts in nude mice.

Notes: The microvessel density was assessed using a monoclonal mouse anti-human CD31 antibody, cellular apoptosis was detected by TUNEL assay using an in situ apoptosis detection kit, and the proliferation was evaluated using immunostaining of PCNA with a monoclonal primary mouse anti-human PCNA antibody. **(A)** Representative tumor sections stained with an anti-CD31 antibody from different treatment groups, **(B)** bar graph to show the effect of treatment with PBS, MSCs-GFP, Lenti-NK4, or MSCs-NK4 on intratumoral microvessel density in nude mice, nude mice treated with MSCs-NK4 had lowest intratumoral microvessel density among all treatment groups, **(C)** representative tumor sections stained with TUNEL where brown cells indicated by arrows are apoptotic cells, **(D)** bar graph to show the effect of treatment with PBS, MSCs-GFP, Lenti-NK4, or MSCs-NK4 on the apoptosis of tumor cells in vivo, nude mice treated with MSCs-NK4 had highest apoptotic index among all treatment groups, **(E)** representative tumor sections stained with anti-PCNA antibody, and **(F)** effect of treatment with PBS, MSCs-GFP, Lenti-NK4, or MSCs-NK4 on the proliferation of tumor cells in nude mice, nude mice treated with MSCs-NK4 had lowest proliferation index among all treatment groups. * $P < 0.05$, by one-way ANOVA.

Abbreviations: PBS, phosphate-buffered saline; MSCs, mesenchymal stem cells; GFP, green fluorescent protein; hpf, high power field; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; PCNA, proliferating cell nuclear antigen; ANOVA, analysis of variance.

xenografts ($7.31\% \pm 1.9\%$) compared to mice treated with PBS ($P < 0.05$ by one-way ANOVA, Figure 6C and D). The apoptotic index in Lenti-NK4 treated tumor xenografts was also higher ($4.2\% \pm 1.3\%$) compared to PBS or MSCs GFP treated mice.

Tumor cell proliferation was evaluated as the percentage of PCNA-positive cells, and no significant differences in the number of PCNA-positive cells in tumors were observed between MSCs-NK4 or Lenti-NK4 treated mice and control groups ($P > 0.05$ by one-way ANOVA; Figure 6E and F). These results indicate that MSCs-NK4 effectively inhibited the growth of gastric cancer xenografts by suppressing tumor-associated angiogenesis and inducing tumor cell apoptosis.

Discussion

Tyrosine kinase receptors are interesting candidates for targeted cancer therapy, as they are often aberrantly activated in human cancers. Among these receptors with tyrosine kinase activity, Met is a “master gene” controlling the growth and invasion of cancer cells and protection from apoptosis and angiogenesis in the tumor microenvironment.^{28,32,50} Met can also be genetically selected for the long-term maintenance of the primary transformed phenotype, and some tumors such as gastric cancer appear to be dependent on (or “addicted” to) sustained Met activity for their growth, survival, and invasion. Because of its dual role as an adjuvant, pro-metastatic gene for some tumor types and as a necessary oncogene for others, Met is a versatile candidate for targeted cancer therapy.⁵¹ To date, a number of drugs has been developed, including chemical kinase inhibitors and monoclonal antibodies targeting either the ligand HGF or the receptor Met.²⁸ In this study, we investigated whether systemic administration of MSCs transduced with NK4 (an antagonist of Met) using lentiviral vectors could inhibit the growth of gastric cancer xenografts in nude mice. First, we isolated and cultured human MSCs, and these MSCs expressed CD44 and CD105 but without CD45 and CD34 expression. An *in vitro* cell migration assay showed that the isolated MSCs could migrate preferably to gastric cancer cells, but human fibroblasts did not exhibit this characteristic. Next, we stably transduced the MSCs with lentiviral vectors carrying *NK4* or GFP. *NK4* was expressed in the conditioned medium and MSC lysates. We then established gastric cancer xenografts in nude mice and treated these mice with MSCs or lentiviral vectors carrying *NK4* cDNA for up to 28 days. The data showed that systemic MSCs-NK4 injection significantly inhibited the growth of gastric cancer xenografts. The microvessel density of tumor

xenografts was decreased, but tumor cell apoptosis was significantly increased in nude mice treated with MSCs-NK4 compared to PBS treated mice. The current data suggest that MSC-delivered *NK4* gene therapy was better than lentiviral vectors carrying *NK4* cDNA for the suppression of gastric cancer xenografts and that *NK4* gene therapy inhibited angiogenesis of tumor xenografts and induced apoptosis of tumor cells. Future mechanistic and clinical studies using MSC-based gene therapy are warranted to test the usefulness and safety of MSCs as a gene delivery vehicle for human gastric cancer therapy.

Gene and cellular therapy has become a promising strategy in the treatment of human cancer in recent years.^{7,22,52,53} However, although significant advances were made for gene therapy of human cancers, major hurdles continue to limit its potential clinical use, eg, tumor tropism of vectors and stimulation of host immune response. In particular, viral vectors are associated with immune recognition, mutagenic integration of viruses into the human genome, and inflammatory toxicity and rapid clearance by liposomes.⁵⁴ Thus, a better gene delivery vehicle could help advance this field. In this regard, MSCs are particularly attractive because they have tumor-targeting properties and are easily isolated, expanded in culture, and genetically manipulated using currently available molecular techniques.^{15–17,20,21,55–59} Moreover, MSCs expressing transgenes can be maintained for a long period of time (up to 3 months) *in vivo* because of their hypo-immunogenic properties and production of immunosuppressive molecules.^{60–62} Thus, MSCs are suggested to have a potential clinical use for cancer gene therapy. At present, various vectors are available to transduce genes into MSCs, including retroviral, adenoviral, and lentiviral vectors.^{16,22,59} Adenoviral vectors are the most commonly used vectors with high titers. However, the transduction efficiency of MSCs by conventional adenoviral vectors has been shown to be only up to 20% because of the low expression of Coxsackie-adenoviral receptors in MSCs.^{57,63} In contrast, retroviral vectors can integrate into the host genome and permit stable or long-term transgene expression. However, a major drawback of retroviral vectors is that they exclusively transduce dividing cells, and the majority of tumor cells do not divide within a given treatment window.⁶⁴ In addition, lentiviral vectors have the ability to transduce both dividing and non-dividing cells as well as to integrate into the genome of target cells, which guarantees long-term expression of the therapeutic genes.^{65,66} Lentiviral vectors have been successfully applied to generate potent dendritic cell based anticancer vaccines and to deliver cancer-specific receptors to T-cells.⁶⁶ In our current study, we transduced MSCs using

lentiviral vectors carrying NK4, and these MSCs were able to infiltrate tumor lesions and self-renew on site. Thus, these cells exhibited better antitumor activity than that of directly delivered plasmids or viral vectors.

Furthermore, in our current study, we isolated human MSCs for in vitro expansion. These MSCs expressed several typical cell surface markers including CD44 and CD45. Moreover, the phenotype of MSCs was not altered after transduction of NK4, indicating that introduction of the *NK4* gene into MSCs by the lentiviral vector did not change MSC morphology and function, consistent with the results of our previous study.⁴¹ MSCs can also achieve a high gene transduction efficiency (>85%) 3 days after exposure to lentiviral vectors. Expression of NK4 was detected in both MSCs-NK4 and the conditioned medium, suggesting that MSCs-NK4 can stably express and continually secrete NK4. Most importantly, MSCs can respond to inflammatory cytokine and chemokine signaling at the site of injury by migrating, proliferating, and differentiating to aid in wound repair.^{11,67} Through the release of a constant stream of inflammatory signals, tumors can be characterized as “wounds that never heal”.¹³ Tumors release inflammatory cytokines and chemokines with the ability to recruit supportive host cells and the potential to activate and recruit MSCs.^{68,69} Thus, MSCs engineered to express tumor killing proteins could be used to destroy tumors and prevent their invasion and metastases. Indeed, our previous study showed that MSCs can migrate to gastric cancer cells in vitro and in vivo.⁷⁰ In the current study, we found that MSCs migrated to gastric cancer cells but not to normal cells in vitro and were present in tumor xenografts in nude mice, indicating that MSCs are a useful and safe vehicle for gene therapy.

In addition, our current study compared MSC-based NK4 gene therapy with lentiviral vectors carrying NK4. We found that MSC-based NK4 gene therapy had better effects on the inhibition of tumor xenograft growth than did direct injection of recombinant Lenti-NK4. Indeed, a previous study³⁹ showed that in the Colon-26 (C-26) lung metastasis model, MSC-based NK4 gene therapy inhibited progression of multiple lung metastases and prolonged survival. In contrast, high-dose recombinant Ad-NK4 (2.0×10^9 infectious units [ifu]/mouse) reduced the numbers of metastatic tumors but did not improve survival. This was probably due to the severe liver damage caused by the adenoviruses. However, low doses of recombinant Ad-NK4 (5.0×10^7 ifu/mouse) had no antitumor effect.³⁹ Adenovirus-mediated induction of high levels (10^9 ifu/mouse) of circulating NK4 significantly inhibited in vivo tumor growth and distant metastasis.³⁶ Therefore, it is possible that the dose

of recombinant Lenti-NK4 (3×10^7 transduction units/mouse, equivalent to lentiviral vector-infected MSCs) delivered in our current study was less than the effective concentration needed. Lentiviral vectors have no tropism to tumor lesions and may not reach the required high levels at the tumor site, thus leading to the poor antitumor effect observed in this study.

NK4-induced anti-angiogenic effects are reproduced in various types of cancer.^{37,71–76} Our current data show that the microvessel density in tumor xenografts treated with MSCs-NK4 was lowest among all treatment groups of mice. Moreover, the cancer cell apoptosis was obviously increased in MSCs-NK4 treated mice. However, our current study is only proof-of-principle, and more research is needed before these findings can be translated into the clinical use of MSCs as a suitable and safe delivery vehicle for gene therapy. It would be interesting to check whether MSC-based NK4 gene therapy could block Met-mediated signaling and suppress the activities of downstream signaling molecules. In future studies, we will further test the efficacy and safety of MSCs as the gene delivery vehicle for cancer treatment in animals and then isolate MSCs from patients for in vitro expansion and gene transduction before injection back into the patients.

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Disclosure

The authors declare no conflicts of interest in this work.

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