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# KAI1/CD82 Genetically Engineered Endothelial Progenitor Cells Inhibit Metastasis of Human Nasopharyngeal Carcinoma in a Mouse Model

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Statistical Analysis C  
Data Interpretation D  
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**Background:** Endothelial progenitor cells (EPCs) are regarded as promising targeted vectors for delivering therapeutic genes or agents in cancer therapy. The purpose of this study was to investigate the role of intravenously administered KAI1/CD82 genetically transduced EPCs in the tumorigenesis and metastasis of nasopharyngeal carcinoma (NPC).

**Material/Methods:** EPCs were isolated from human umbilical cord blood, expanded in culture, and stably transduced with lentiviral vectors expressing KAI1/CD82. The KAI1/CD82 EPCs were injected intravenously into nude mice bearing human NPC xenografts. Tumor growth and the incidence of liver and lung metastases were observed. Expression of KAI1/CD82 was determined by immunofluorescent staining.

**Results:** The NPC model was successfully established. Tumor growth was not suppressed when mice were injected with KAI1/CD82 EPCs (KAI1/CD82 EPCs group) compared with when non-transduced EPCs was present (EPCs group) or the control ( $1.485 \pm 0.234$ ,  $1.388 \pm 0.204$ , and  $1.487 \pm 0.223$ g, respectively;  $P > 0.05$ ). However, the incidence of lung metastasis was significantly reduced in the KAI1/CD82<sup>+</sup> EPCs group compared with the EPCs group and the control group (10%, 55% and 45%, respectively;  $P = 0.005$ ), and there was a significant decrease in the number of metastatic foci on the lung surface ( $17.50 \pm 3.54$ ,  $34.27 \pm 5.35$ , and  $38.44 \pm 9.63$  respectively;  $P = 0.007$ ). Moreover, KAI1/CD82 was expressed in lung metastatic foci of the KAI1/CD82 EPCs group, but not in the EPCs group and control group.

**Conclusions:** EPCs can be used as a delivery vehicle for suppressor genes KAI1/CD82 to NPC, and the migration of KAI1/CD82 genetically engineered EPCs can inhibit NPC lung metastasis in a mouse model.

**MeSH Keywords:** **Antigens, CD82 • Genes, Tumor Suppressor • Lymphatic Metastasis • Nasopharyngeal Neoplasms**

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## Background

Nasopharyngeal carcinoma (NPC) is a malignant neoplasm that has a remarkable ethnic and geographic distribution, with a high annual incidence rate of approximately 20–50 cases/100 000 people [1]. Although the combination of chemotherapy and intensity modulated radiotherapy has improved 5-year the overall survival rate of NPC patients, the overall prognosis remains poor [2–4]. Advanced and metastatic NPC results in resistance to conventional treatment, which is often fatal. Discovery of novel molecular targets for effective prevention and treatment of NPC remains a major concern. KAI1/CD82 is regarded as a metastatic suppressor gene, which has been found to be involved in the invasion and metastasis of some human cancers [5–7]. Little work on KAI1/CD82 has been done on this gene in NPC, and its role in the metastasis of NPC remains elusive [8].

Endothelial progenitor cells (EPCs) are a subpopulation of pluripotent hematopoietic stem cells, which possess the ability to proliferate, migrate, colonize, and differentiate into endothelial lineage cells [9]. Many studies demonstrated that EPCs play an important role in tumor angiogenesis. Bone marrow circulation-derived EPCs, as a specific host of tumor tissue, are involved in the process of vascular growth and promote tumor growth [10–12]. There is great interest in evaluating the role of EPCs in tumor angiogenesis, metastasis, and drug therapy monitoring. Based on these characteristics, EPCs are regarded as favorable carriers or vehicles to deliver the therapeutic gene for cancer gene therapy [13,14].

Herein, we administrated human CD82/KAI1 EPCs intravenously to nude mice transfected with human NPC cell line CNE-2Z to explore whether CD82/KAI1 genetically-transduced EPCs therapy results in inhibition of tumorigenesis and metastasis of NPC.

## Material and Methods

### Animals

Sixty 4–6-week-old female BALB/c-nu mice were purchased from the Sikerui Biotechnology Co, Ltd. (Nanjing, China; certificate number SCXK -2011-0003). Procedures involving animals and their care were conducted in conformity with NIH guidelines (NIH Pub. No. 85-23, revised 1996) and were approved by the Animal Care and Use Committee of Bengbu Medical College. Animals were maintained under controlled conditions (room temperature 22–26°C; relative humidity 50–60%; 12-h light/dark cycle) and had free access to food and water.

### CNE-2Z culture

CNE-2Z human NPC cell line cells (Sinobest Biotechnology, Shanghai, China) were cultured under standard humidified conditions (37°C, 5% CO<sub>2</sub>) with RPMI1640 Medium (Sangon, Shanghai, China) supplemented with 10% fetal bovine serum (Senrui Biotechnology, Tianjin, China), and 1% penicillin/streptomycin (North China Pharmaceutical Group Corporation). At 80% confluence, CNE-2Z cells were washed with PBS and then treated with 0.05% trypsin for 3 min for digestion.

### EPCs culture and function analysis

Briefly, EPCs were isolated from human umbilical cord blood and cultured in medium containing M199 supplemented with 200 ml/L FBS, 10 µg/L vascular endothelium growth factor (VEGF; eBioscience, USA), 15g/L bovine pituitary extract, and 10 µg/L basic fibroblast growth factor (bFGF; eBioscience, USA) as previously described [15]. To analyze the function of EPCs, adherent cells were first incubated with 2.4 mg/L of Dil-acetylated low-density lipoprotein (Dil-aCLDL) (MolecularProbe, USA), then stained with FITC-Ulex europaeus lectin-1 (FITC-UEA-I) (Sigma, USA). Cells with double-positive fluorescence were identified as differentiating EPCs.

### Transduction of EPCs with lentiviral vector containing KAI1/CD82 gene

EPCs were seeded onto 24-well plates (1×10<sup>5</sup> cells per well) and were allowed to attach. On the second day, the culture media was refreshed with 6µg/ml polybrene and cells were transduced with lentiviral transfer vector (MOIs greater than 50) carrying the KAI1/CD82 gene (Biomeditech, Shanghai, China) according to the manufacturer's protocol. After 24 h, the media were replaced. The transfection efficiency was detected after 48 h using a fluorescence microscope (Olympus, Japan). After 3 days, the cells were selected with 2.5 µg/µl Puromycin for 6 days. The survival-transfected cells reached more than 90%.

### Cell viability assay

Prior to transplantation, 0.4% trypan blue exclusion was performed to detect the viability of CNE-2Z used for experiments, and the live/dead viability was determined. Cells had to have more than 95% viability.

### In vivo tumor cell implantation and intravenous administration of KAI1/CD82 transduced EPCs

A total of 60 BALB/c nude mice were randomly divided into 3 groups (n=20 in each group). All mice were injected with CNE-2Z cells subcutaneously in a volume of 0.2 ml (a density of 2×10<sup>6</sup>/mL) into the armpits of mice. After 1 week, 20 mice

received non-transduced EPCs injection (EPCs group), 20 mice received KAI1/CD82 EPCs (KAI1/CD82 EPCs group), and the remaining 20 mice received saline as control via the tail vein. The status of mice was clinically checked every day. Mice were sacrificed when there was weight loss and decreased activity and food intake.

### Histologic analysis

The animals were euthanized with 100 mg/kg of pentobarbital administration. The transplanted tumors, liver, and lung tissue were removed, fixed in Bouin's solution, and embedded in paraffin to detect metastases. The metastatic foci were observed under a microscope and measured as the diameter of the foci (Grade I, <0.15 mm; Grade II, 0.15–1 mm; Grade III, 1–2 mm; Grade IV, >2 mm). The total number (n) of metastatic foci was calculated as (Grade I n) + (Grade II n)×2+ (Grade III n)×3 + (Grade IV n)×4.

### Immunophenotype analysis

EPCs or tissue sections from mice were fixed with 4% paraformaldehyde (Sigma, USA) for 30 min in room temperature and washed in 20 mM phosphate-buffered saline (PBS) followed by permeabilization for 30 min with 0.4% Triton-X (Sigma, USA) in PBS. To inhibit the endogenous peroxidase activity, cells were treated with 3% H<sub>2</sub>O<sub>2</sub> for 15 min, and blocked with 10% normal goat serum for 10 min. Then, cells were incubated with rabbit polyclonal anti-human CD31 antibody (Boao Biotechnology, Beijing, China; 1: 50), von Willebrand factor (vWF) antibody (Boao Biotechnology, Beijing, China; 1: 50), or KAI1/CD82 (BD Bioscience, USA; 1: 500) overnight at 4°C. Cells were washed with PBS 3 times and then incubated with a biotinylated goat anti-rabbit IgG or goat anti-mouse, following the SP Kit (Zhongshan Biotechnology, Beijing, China) manufacturer's protocol. Antigen-antibody complexes were visualized with 3,3'-diaminobenzidine (DAB) (Noblerlyder Technology, Beijing, China). Cells were observed under a light microscope (Olympus, Japan) and the number of CD31 or vWF positive cells was evaluated.

### Immunoblotting

For Western blot analysis, EPCs were lysed with RIPA buffer containing PMSF and phosphatase inhibitors (Beyotime Biotechnology, Beijing, China) and homogenized by ultrasound. Samples were then centrifuged at 12 000 rpm for 30 min at 4°C. Supernatants were isolated and protein concentration measured using a bicinchoninic acid protein kit (Beyotime Biotechnology, Beijing, China). Samples were mixed with loading buffer (Beyotime Biotechnology, Beijing, China) and boiled at 100°C for 5 min. Equal amounts of protein lysates (20 µg) were loaded and separated by 10% SDS-NuPage gel (Bio-Rad,

USA) and transferred to nitrocellulose membrane (NC) membranes (Baisai Biotechnology Shanghai, China). Membranes were blocked in 5% BSA for 1 h, and then incubated with rabbit monoclonal antibody against human KAI1/CD82 (Anbo Biotechnology, USA; 1: 500) and then β-actin (Sangon, Shanghai, China; 1: 2000) in 5% BSA-TBST overnight at 4°, followed by HRP-conjugated secondary antibodies (1: 3000) in TBST incubated at room temperature for 30 min. Detection was carried out with an ECL plus kit (Pierce, USA). The intensity of bands in Western blots was analyzed with Quantity-one software.

### Statistical analysis

All data are presented as mean ±SD. Statistical analysis was performed using SPSS Statistics 20 Software. Differences between groups were compared with one-way ANOVA followed by the LSD test where appropriate. Categorical variables are expressed as frequencies and percentages, and were analyzed with the Pearson  $\chi^2$  test. *P* values less than 0.05 were considered statistically significant.

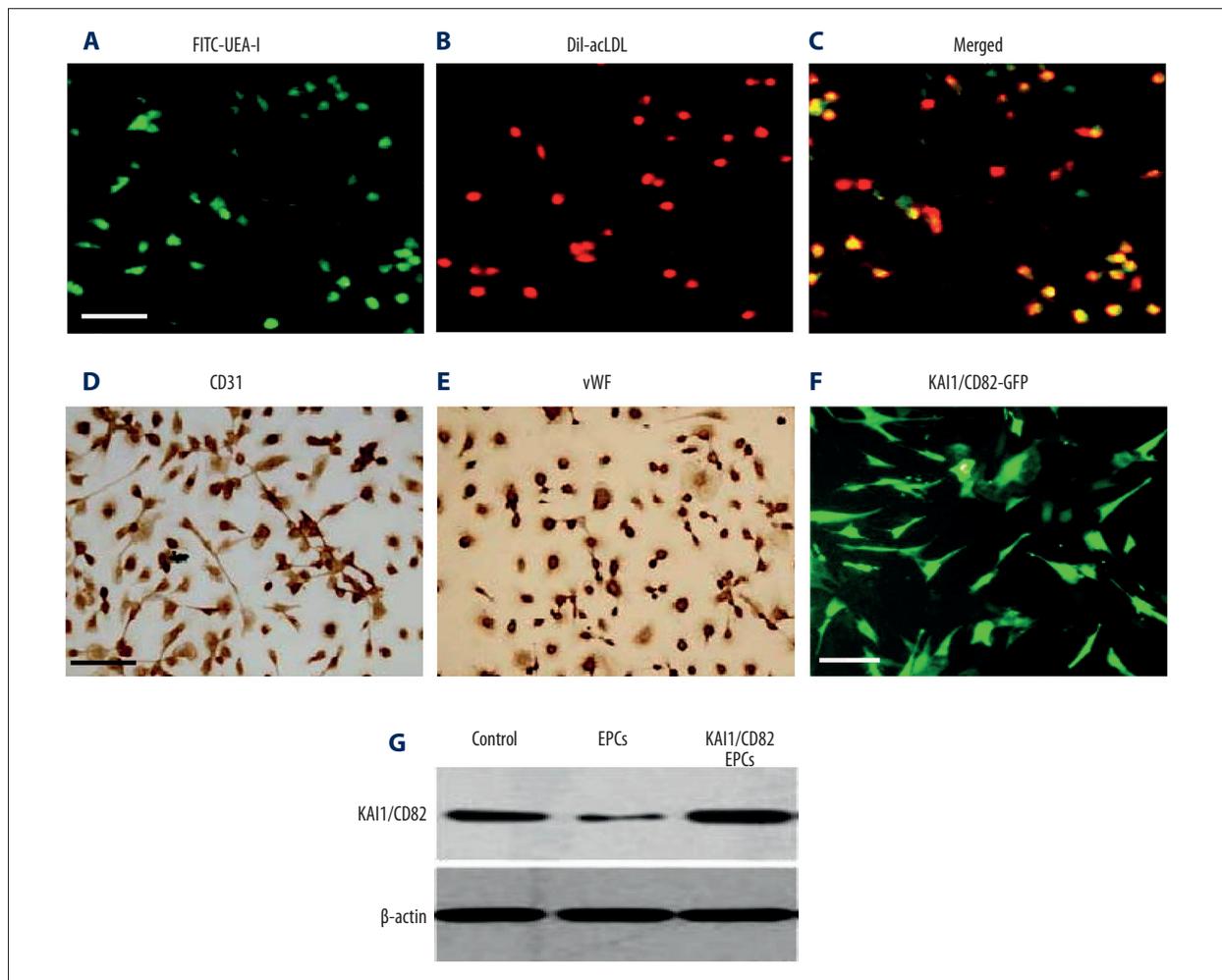
## Results

### Characterization and transduction of KAI1/CD82 gene of EPCs

Fluorescent staining showed that adherent cells positive to both FITC-UEA-I and Dil-acLDL were differentiating EPCs (Figure 1A–1C). Both CD31 and vWF proteins were expressed in the adherent cells, with brown-yellow staining in the cytoplasm (Figure 1D, 1E). The positive rates of CD31 and vWF were 88.9±3.2% and 76.5±4.7%, respectively. EPCs were then transduced with KAI1/CD82-GFP lentivirus and selected with Puromycin. After drug selection for 6 days, the expression of KAI1/CD82 in KAI1/CD82-transduced EPCs was higher than the non-transduced EPCs (see the Western blot result in Figure 1 G).

### Effect of KAI1/CD82 gene transduction on the tumorigenesis of NPC

To investigate the effect of KAI1/CD82 gene transduction on the tumorigenesis of NPC, we first established a human NPC model in mice by implanting CNE-2Z cells and human NPC cells. Seven days after implantation, the selected KAI1/CD82 EPCs cells or non-transduced EPCs were injected i.v. through a lateral tail vein. CNE-2Z cells formed palpable tumors in all mice (Figure 2A), and there was no difference in the tumor onset among the KAI1/CD82 EPCs group, the EPCs group, and the control group (*P*=0.771, Figure 2A, Table 1). No significant differences were observed in the palpable tumor weight among these 3 groups (*P*>0.05, Table 1), indicating KAI1/CD82-transduced EPCs cannot suppress NPC growth.



**Figure 1.** Representative images of double-immunofluorescence staining, bar=100 μm. (A–C) CD31 and vWF expression on EPCs for immunohistochemical staining, bar=100 μm (D, E). Fluorescent images of EPCs cells transfected with lentiviral vector carrying the KAI1/CD82-GFP gene, bar=100 μm (F). Western blot analysis of KAI1/CD82 protein expression among different groups (G).

### Effect of KAI1/CD82 gene transduction on the development of NPC metastasis

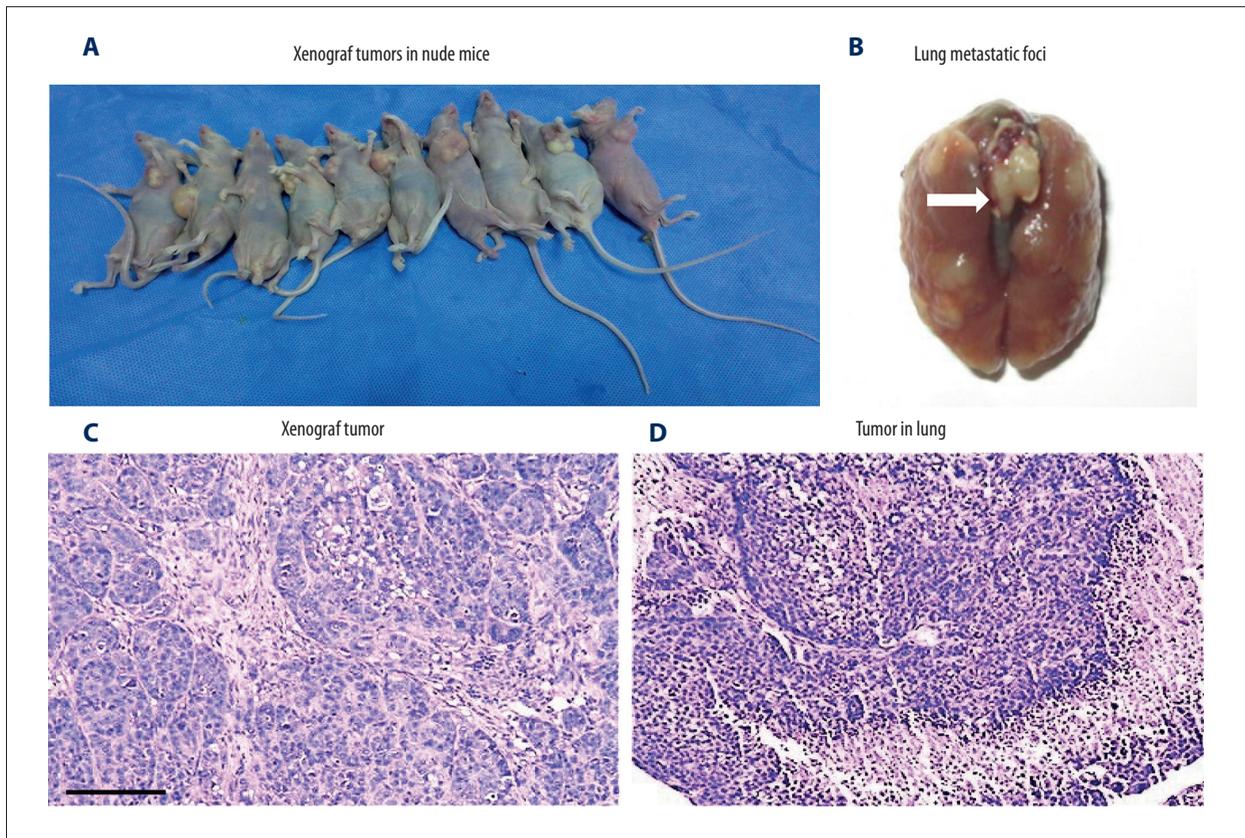
In the present study, NPC with liver metastasis was not found in the NPC nude mice. The incidence of lung metastasis was significantly reduced in the KAI1/CD82 EPCs group (10%) compared with that of the EPCs group (55%) and the control group (45%) ( $P=0.005$ , Table 2, Figure 2B–2D). The total number of metastatic foci on the lung surface significantly decreased in the KAI1/CD82 EPCs group compared to the EPCs group and the control group ( $17.50\pm 3.54$ ,  $34.27\pm 5.35$ , and  $38.44\pm 9.63$ , respectively;  $P=0.007$ ) (Table 1).

The metastatic foci on the lung surface from mice injected with KAI1/CD82 EPCs showed KAI1/CD82 expression using immunohistochemistry (Figure 3A–3C). In contrast, KAI1/CD82 expression was not detected in the metastatic foci of the EPCs

group or the control. Thus, these data indicate that KAI1/CD82-transduced EPCs are a potential effective therapy for inhibiting the incidence and development of lung metastasis in NPC.

### Discussion

Tumor cell invasion and metastasis are the leading causes of cancer mortality and morbidity. NPC is characterized by a high risk of distant metastasis [16]. Advanced and distant metastatic NPC dramatically affect the efficacy of conventional treatment and the survival rate [17,18]. Concentrating research efforts on preventing or controlling metastasis may limit NPC progression and reduce mortality for many cancer patients. However, the optimal treatment of NPC metastasis remains challenging and controversial. Our present findings suggest that EPCs can be used as a delivery vehicle for suppressor genes KAI1/CD82



**Figure 2.** Representative images of xenograft tumor within armpit (A) and tumor developed in the lung (white arrow) (B) in nude mice observed after tail vein injections of CNE-2Z cells. HE staining of paraffin-embedded sections of xenograft tumor (C) and lung tissue (D). The solid tumor mass (blue) was seen, with cohesive sheet and islands of tumor cells with a rich lymphoid stroma, bar=200  $\mu$ m.

**Table 1.** Characteristics of human NPC metastasis to the lung in nude mice.

	Control (n=20)	EPCs group (n=20)	KAI1/CD82 EPCs (n=20)	P value
Incidence of lung MTS (n, %)	9 (45%)	11 (55%)	2 (10%)	0.005
Numbers of lung MTS	38.4 $\pm$ 9.6	34.3 $\pm$ 5.4	17.50 $\pm$ 3.5	0.007

MTS – metastasis. P value tested by chi-square test or one-way ANOVA test.

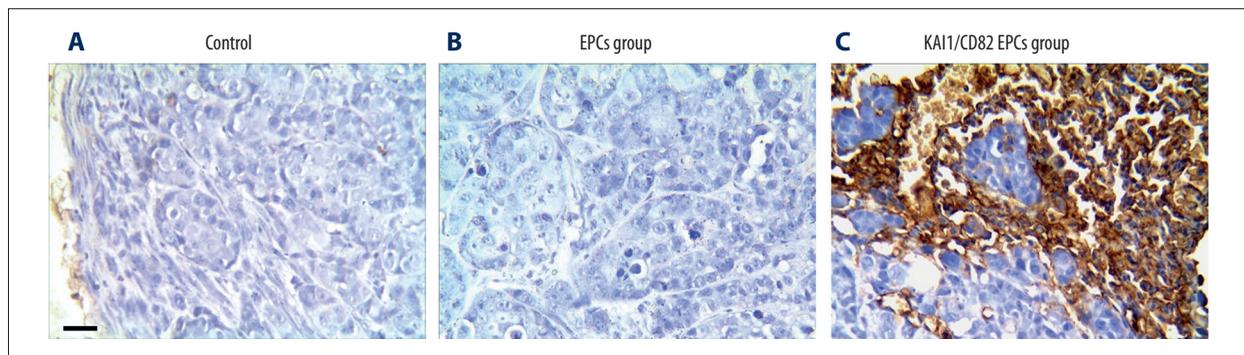
**Table 2.** Tumor onset and weight in the nude mice among different groups.

Group	Tumor onset	Tumor weight (g)
EPCs	14.70 $\pm$ 3.81	1.388 $\pm$ 0.204
Control	14.20 $\pm$ 3.55	1.487 $\pm$ 0.223
KAI1/CD82 EPCs	15.05 $\pm$ 3.85	1.485 $\pm$ 0.224
P value	0.771	0.274

EPC – endothelial progenitor cells. P value tested by one-way ANOVA test.

due to the unique abilities of EPCs to migrate to the sites of tumors, and the migration of EPCs carrying KAI1/CD82 decreased NPC lung metastasis in the human NPC mouse model. In this study, only 10% of NPC mice treated with KAI1/CD82 EPCs developed lung metastasis, which is remarkably lower, and the incidence of metastasis was obviously lower than that of NPC mice treated with EPC (55%) or not (45%).

Recent studies suggest that bone-marrow-derived EPCs are involved in neovessel formation in spontaneous and transplanted tumors and in metastatic tumors in animals and humans [10,12]. The tumor-derived paracrine signals and the tumor-secreted factors, including VEGF, GM-CSF, and osteopontin, instigate the bone marrow and attract the circulating



**Figure 3.** KAI1/CD82 protein expression in lung metastatic foci. KAI1/CD82 protein expression was negative in the control group and EPCs group (A, B), and was strongly expressed in the KAI1/CD82 EPCs group, bar=50  $\mu$ m (C).

EPCs to the tumor sites where they incorporate into neovessels [19–21]. Although the controversy exists about the functional significance of tumor vasculature and growth of EPCs, there has been considerable interest in the application of EPCs as surrogate markers for predicting and monitoring cancer progression, as well as an attractive target for therapeutic intervention [22–26]. Muta et al. found that administration of EPCs-NC promoted Walker256 tumor growth and neovascularization. Conversely, the IL-12 gene-transfected EPCs-NC effectively inhibited tumor angiogenesis because these cells secreted IL-12, activating natural killer cells and cytotoxic T cells [25]. In addition, the majority of studies show that intravenous administration of EPCs carrying tumor-targeted genes can arrive at the site of tumor rapidly and in high concentration [27–29]. Therefore, gene-transduced EPCs could be useful as a tumor-specific drug delivery system.

In the present study, the KAI/CD82-transduced EPCs were proved to be in the lung metastasis site where KAI1/CD82 was positive and progressively decreased the incidence of NPC, but KAI1/CD82 was absent in the NPC metastasis with non-transduced EPCs treatment. Studies have indicated that KAI/CD82

was correlated with tumor invasion, differentiation, TNM stage, distant metastasis, and lymph node metastasis [5,8,30,31]. Wang et al. reported that KAI1/CD82 expression decreased with the advancing tumor stage and lymph nodes metastasis [8]. Moreover, KAI1/CD82 expression was lost in the NPC cells with high metastatic potential, and KAI1/CD82 gene was expressed at low levels in NPC tissue, while high expression was identified in non-neoplastic nasopharyngeal tissues, which is consistent with our results. Overall, KAI1/CD82 expression is remarkably associated with NPC metastasis, and may be a new method for the development of gene therapy and for predicting NPC progression, metastasis, and prognosis.

## Conclusions

Our observations suggest that EPCs are able to carry and express KAI1/CD82 in nasopharyngeal carcinoma following intravenous administration. KAI1/CD82 genetically engineered EPCs can inhibit lung metastases of human nasopharyngeal carcinoma. KAI1/CD82 has potential for predicting progression, metastasis, and prognosis of NPC.

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