

***In vivo* knockdown of CXCR4 using jetPEI/CXCR4 shRNA nanoparticles inhibits the pulmonary metastatic potential of B16-F10 melanoma cells**

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Abstract. Metastasis is a key factor that limits survival in the majority of patients with cancer. Thus, numerous efforts have been made to elucidate the molecular mechanisms involved in this phenomenon. B16-F10 melanoma cells have been demonstrated to be highly metastatic to the lungs in mice. The aim of the current study was to investigate the role of CXC motif chemokine receptor 4 (CXCR4) in the metastatic potential of B16-F10 melanoma cells in mice. *In vitro* transfection of B16-F10 tumor cells with CXCR4 short hairpin RNA (shRNA) expressing plasmids (CXCR4 shRNA) significantly reduced the expression levels of CXCR4 mRNA (80%) and protein (68%), compared with the control. In addition, these results demonstrated that pulmonary metastasis was significantly inhibited (85%) in mice inoculated with CXCR4 shRNA-transfected B16-F10 melanoma cells. The polycation-based nanoparticle (jetPEI) was used to investigate the effect of CXCR4 knockdown *in vivo* on the metastatic potential of B16-F10 melanoma cells. The number of pulmonary metastatic nodules was significantly reduced (50%) in animals that received a retro-orbital injection of jetPEI-CXCR4-1 shRNA. The current study demonstrated that CXCR4 serves a role in the metastatic potential of B16-F10 melanoma cells. Currently there is a great interest in the development of antagonists for the therapeutic targeting of CXCR4 expression. Taking the results of the current study and the fact that CXCR4 is highly conserved between humans and mice into account, this experimental model of metastasis with B16-F10 melanoma cells

may aid in the discovery of CXCR4 antagonists with clinical implications.

Introduction

Metastasis is a key factor that limits survival in the majority of patients with cancer. The metastatic potential of malignant melanoma is considerably greater than that of other primary solid tumors (1). The incidence of malignant melanoma has increased during recent decades, with a high mortality rate due to the metastatic dissemination of tumor cells (2).

The molecular mechanisms involved in melanoma metastasis have been thoroughly investigated however remain to be fully understood. It is known that chemokines mediate numerous physiological and pathological processes associated with cell homing and migration (3). The CXC motif chemokine receptor 4 (CXCR4) is a transmembrane receptor that belongs to the chemokine receptor family (4). CXCR4 was initially reported to mediate homing of leukocytes into tissues that produce its ligand, stromal cell-derived factor 1, also known as CXC motif chemokine 12 (CXCL12) (5,6). It has been reported that CXCR4 is the most widely expressed chemokine receptor in numerous different types of cancer, suggesting that this receptor is additionally involved in the migration of tumor cells (2). In this context, it was observed that the expression of CXCL12 is elevated in common sites of metastasis including the lungs, liver, lymph nodes and bone marrow, suggesting that the CXCL12/CXCR4 axis serves a key role in the metastatic destination of numerous types of human cancer (3,4,7).

Numerous previous studies have demonstrated that CXCR4 serves an important role in the metastasis in numerous types of cancer, including human melanoma (1,8-10). It has been reported that CXCL12 binds to CXCR4, which through multiple divergent pathways, leads to cell adhesion, invasion, cell survival and angiogenesis (11-13).

The murine B16-F10 melanoma cell line is highly metastatic to the lungs, and these tumor cells have been used to investigate the molecular mechanisms involved in metastasis (14,15). However, the role served by CXCR4 in this experimental model

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of metastasis remains to be elucidated. Thus, Chung *et al.* (16) observed that the treatment of mice with fucoxanthin inhibits CXCR4, CD44 and matrix metalloproteinase-9 (MMP-9) expression in B16-F10 melanoma cells, which is accompanied by the reduction of lung metastasis. Whilst CD44 and MMP-9 are additionally involved in metastasis, the precise role of CXCR4 in this phenomenon remains unclear in B16-F10 cells. A previous study demonstrated that micro-RNA-199a-5p regulates the expression of 15 metastasis-associated genes in B16-F10 melanoma cells, including CXCR4 (10). However, the current study did not investigate the specific role of CXCR4 in this experimental model of metastasis.

RNA interference (RNAi) is a powerful gene silencing technology with specificity, high efficiency and low toxicity (17,18). Results from phase I human studies are promising, suggesting that RNAi-based cancer therapy may be a potential alternative strategy for the treatment of cancer (19). In a previous study, the role played by the RNA-dependent protein kinase in the growth and metastasis of B16-F10 melanoma cells was investigated using RNAi (20,21).

In the present study, RNAi technology was used to investigate the effect of CXCR4 knockdown on the pulmonary metastasis of B16-F10 melanoma cells in mice. To address this question, B16-F10 melanoma cells were transfected with CXCR4 short hairpin RNA (shRNA)-expressing plasmids, and were subsequently intravenously injected into mice. Additionally, the effect of the intravenous injection of polycation-based nanoparticles (jetPEI/CXCR4 shRNA) on the pulmonary metastasis of B16-F10 melanoma cells was investigated in animals that had previously received an intravenous inoculation of B16-F10 tumor cells. The effects of CXCR4 knockdown *in vitro* and *in vivo* were evaluated by counting the number of metastatic nodules in the lungs of mice.

Materials and methods

Animals. All protocols involving animals were reviewed and approved by the Institutional Animal Care Committee. A total of 200 C57BL/6 mice (6-week-old; 20–25 g; 10 per group) raised at the Central Animal Laboratory of Ribeirão Preto School of Medicine (University of São Paulo; São Paulo, Brazil) were used for the current study. All protocols involving animals were reviewed and approved (certificate no. 062/2006) by the Animal Care Committee of the Ribeirão Preto Medical School.

B16-F10 melanoma cell culture conditions. B16-F10 melanoma cells (Ribeirão Preto Medical School, University of São Paulo, São Paulo, Brazil) were maintained in Roswell Park Memorial Institute 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% inactivated fetal calf serum (Invitrogen; Thermo Fisher Scientific, Inc.), 2 mM L-glutamine (Invitrogen, Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (100 U/ml; Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere at 37°C and 5% CO₂.

Construction of CXCR4 shRNA expression vector. A total of two shRNA target sequences were selected from different

positions within the mouse CXCR4 cDNA sequence (GenBank, accession no. BC031665) corresponding to nucleotides 85–103 (CXCR4-1 shRNA) and 409–427 (CXCR4-2 shRNA). The shRNA sequences are presented in Table I.

These target sequences were BLAST searched (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to ensure that only the CXCR4 gene was targeted. The target sequence of the negative control group used as the control shRNA had no homology with that of humans or mice. The hairpin loop region was annealed with its complementary strand and was cloned into the psiSTRIKE vector controlled by the Pol III U6 promoter (U6 Hairpin Cloning Systems; Promega, Madison, WI, USA) according to the manufacturer's instructions. The screening for the inserts was by digestion with Pst I (Promega).

In vitro transfection. B16-F10 melanoma cells were plated in tissue culture flasks at a density of 7×10⁵ cells. Following an overnight incubation and when ~70–80% confluent, the cells were transfected with 30 µg CXCR4 shRNA and 30 µl Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at a v/w ratio of liposomes to shRNA of 1:1. The transfection efficiency (~75–80%) was evaluated using a green fluorescent protein (GFP) expression plasmid (Invitrogen; Thermo Fisher Scientific, Inc.). Prior to the *in vivo* study, the effectiveness of the two plasmid-based CXCR4-specific shRNAs (CXCR4-1 shRNA and CXCR4-2 shRNA) in reducing the CXCR4 expression in cultured B16-F10 cells was investigated. Subsequently, tumor cells were transfected with CXCR4-1, CXCR4-2 or control shRNA for 5 h, following which the cells were washed, suspended in medium and maintained in culture for 24 or 48 h at 37°C. To determine the CXCR4 mRNA and protein levels, lysates of the B16-F10 melanoma cells were used for RNA isolation and western blot analysis.

RNA isolation. Total cellular RNA was extracted using TRIzol-LS Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The integrity of RNA was assessed using an Agilent Bioanalyzer (Agilent 2100; Agilent Technologies, Inc., Santa Clara, CA, USA).

Analysis of CXCR4 mRNA expression. Reverse transcription-polymerase chain reaction (RT-PCR) was conducted with 1.2 µg total RNA and 0.5 µg Oligo(dT) primer (Invitrogen; Thermo Fisher Scientific, Inc.), 1 U reverse transcriptase (Promega), 1 U RNase inhibitor (Invitrogen; Thermo Fisher Scientific, Inc.), 5 ml 5X M-MLV RT 5X reaction buffer (Promega) and 4 ml MgCl₂ (Promega). The β-actin gene was used as a reference control gene to normalize the expression value of CXCR4. The primers used for CXCR4 were as follows: Sense, 5'-ACAGGTACATCTGTGACCGCCTTT-3' and anti-sense, 5'-TGCTCTCGAAGTCACATCCTTGCT-3' (Invitrogen; Thermo Fisher Scientific, Inc.; GenBank accession no. BC031665). The oligodeoxynucleotide primers for β-actin used for amplification were sense 5'-TGGAAATCCTGTGGCATCCATGAAAC-3' and anti-sense 5'-TAAACGACGCTCAGTAACAGTCCG-3' (Integrated DNA Technologies, Coralville, IA, USA; GenBank Accession No. BC014861) PCR conditions for CXCR4 were as follows: Denaturation for 5 min at 94°C followed by 35 cycles of 1 min at

Table I. Sequences of shRNA oligonucleotides.

shRNA	Target position	Sequence
CXCR4-1	85-103	5'-ACCGCGATCAGTGTGAGTATATAAAGTTCTC TTATATACTCACACTGATCGCTTTTTC-3'
CXCR4-2	409-427	5'-ACCGGTAAGGCTGTCCATATCATAAGTTCTCT ATGATATGGACAGCCTTACCTTTTTC-3'
Control	-	5'-ACCGAAGCGCTGCCGCGACGTTGAAGTTCTCT CAACGTCGCGGCAGCGCTTCTTTTTC-3'

Reference sequences for target positions: BC031665. shRNA, short hairpin RNA; CXCR4, CXC motif chemokine receptor 4.

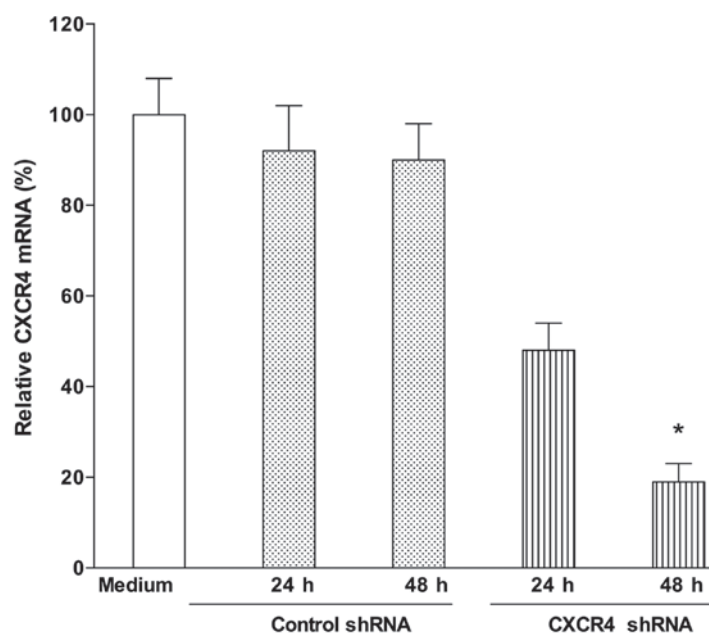


Figure 1. CXCR4 mRNA expression in B16-F10 melanoma cells following transfection with CXCR4 shRNA plasmids. The level of CXCR4 mRNA was determined by reverse transcription-polymerase chain reaction and β -actin was used as the control. The level of CXCR4 mRNA in B16-F10 melanoma cells incubated with Roswell Park Memorial Institute 1640 medium was set as 100%. Values are presented as the mean \pm standard deviation of three independent experiments, and the bars are representative of one typical experiment. * $P < 0.001$, vs. control. CXCR4, CXC motif chemokine receptor 4; shRNA, short hairpin RNA.

94°C, 1 min at 51°C and 1 min at 72°C, then 10 min extension at 72°C in a thermocycler (PCR-Sprint ThermoHybrid; Abgene; Thermo Fisher Scientific, Inc.). PCR products of β -actin (364 base pairs) and CXCR4 (291 base pairs) were analyzed by electrophoresis in a 1.5% agarose gel (Sigma-Aldrich, St. Louis, MO, USA) and visualized using UV fluorescence following staining with ethidium bromide (Merck Millipore, Darmstadt, Germany). Quantification of CXCR4 bands was performed by using ImageQuant software, version 3.3 (GE Healthcare Life Sciences, Chalfont, UK) and the results were expressed as a percentage of the control.

Western blot analysis. B16-F10 adherent cells were detached using ethylenediaminetetraacetic acid (Merck Millipore) and the cell pellet, obtained following centrifugation (1,200 \times g for 10 min at 25°C) was resuspended in 300 μ l phosphate-buffered saline containing 0.1% aprotinin, 0.1% leupeptin and 1% Triton X-100 (Sigma-Aldrich). Protein concentration was determined by the Cadman method (22). Total cellular protein (30 μ g) was

separated by electrophoresis through 10% SDS-PAGE (Merck Millipore), and proteins were transferred onto nitrocellulose membranes (GE Healthcare Life Sciences). The membranes were blocked with 10% dried milk, washed with Tris-buffered saline with 0.1% Tween-20 for 20 min and subsequently incubated with 1:250 rabbit anti-CXCR4 polyclonal antibodies (H-118; cat. no. sc-9046; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 90 min at room temperature. Anti-rabbit antibodies conjugated to horseradish peroxidase (GE Healthcare Life Sciences) were added (1:500) and the membranes incubated at room temperature for 60 min under agitation. Antibody labeled protein bands were visualized using enhanced chemiluminescence detection reagents (GE Healthcare Life Sciences) applied following the manufacturer's instructions. Anti- β -actin antibody (I-19; cat. no. sc-1616; goat polyclonal; 1,400; Santa Cruz Biotechnology, Inc.) were used as a control. Quantification of bands was performed using ImageQuant software, version 3.3 (GE Healthcare Life Sciences) and the results were expressed as a percentage of the control.

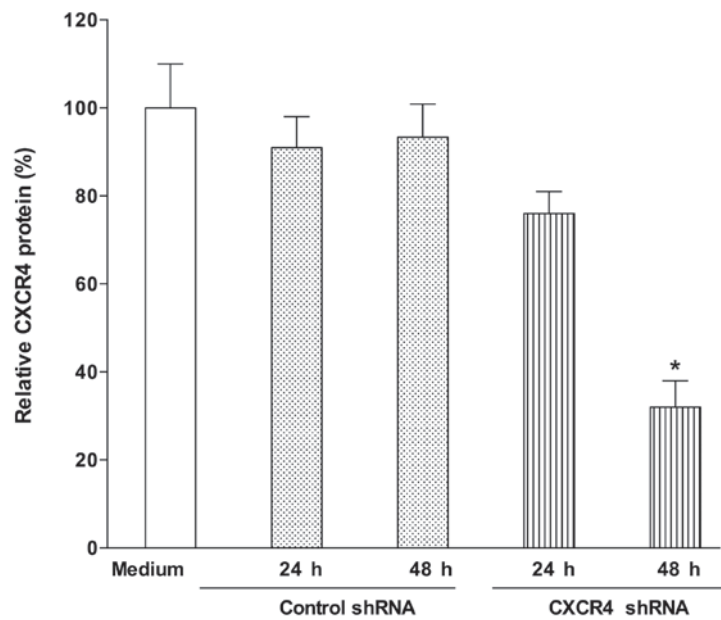


Figure 2. Protein expression levels of CXCR4 in the B16-F10 melanoma cells following transfection with CXCR4 shRNA plasmids, measured by western blot analysis. β -actin was used as a loading control. The level of CXCR4 protein in B16-F10 melanoma cells incubated with Roswell Park Memorial Institute 1640 medium was set as 100%. Values are presented as the mean \pm standard deviation of three independent experiments, and the bars are representative of one typical experiment. * $P < 0.001$, vs. control. CXCR4, CXC motif chemokine receptor 4; shRNA, short hairpin RNA.

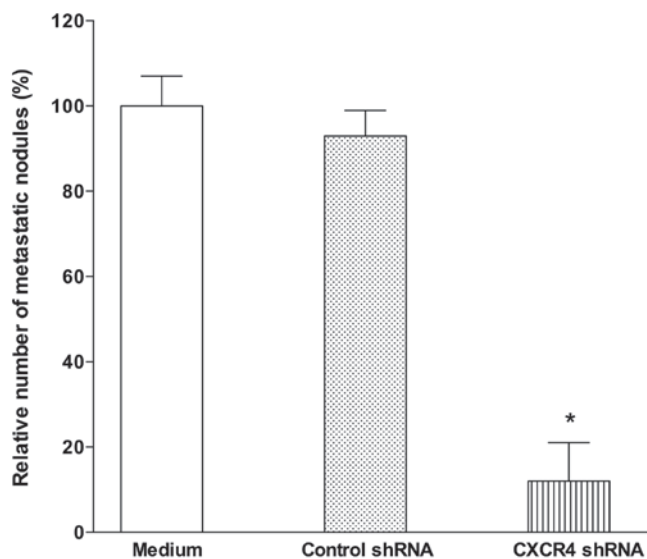


Figure 3. Effect of CXCR4 shRNA on the pulmonary metastatic progression potential of B16-F10 melanoma cells. $n=10$. The animals were sacrificed on day 21 and pulmonary metastatic nodules were counted using a dissecting microscope. Values are presented as the mean \pm standard deviation. Data of one representative experiment of three conducted are presented. * $P < 0.001$, vs. control. CXCR4, CXC motif chemokine receptor 4; shRNA, short hairpin RNA.

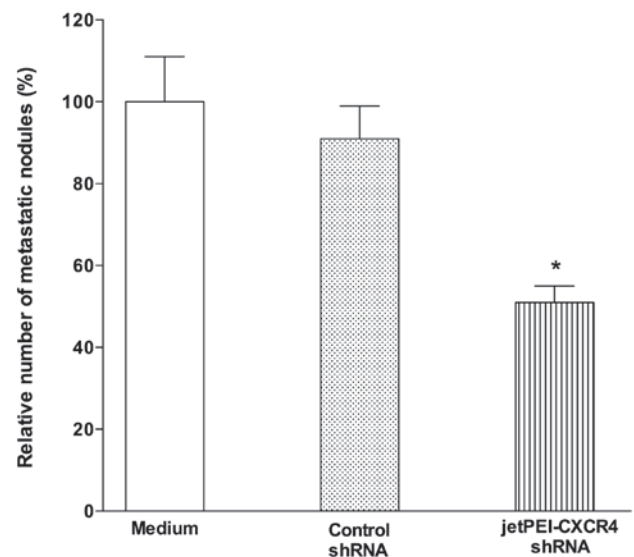


Figure 4. Effect of retro-orbital injection of jetPEI/CXCR4 shRNA on the pulmonary metastatic progression potential of B16-F10 melanoma cells. $n=10$. Values are presented as the mean \pm standard deviation. Data of one representative experiment of three conducted are presented. * $P < 0.001$, vs. control. CXCR4, CXC motif chemokine receptor 4; shRNA, short hairpin RNA.

Retro-orbital injection of transfected B16-F10 melanoma cells into mice. For the *in vivo* experiments, B16-F10 melanoma cells were transfected with CXCR4-1 shRNA or control shRNA for 5 h, following which the cells were washed in RPMI, suspended in medium (RPMI) and maintained in culture for 24 h prior to injection into the mice. The viability of cells was assessed by trypan blue staining (Sigma-Aldrich) and was $>95\%$. The mice were randomly separated into groups of 10 animals. Each group of C57BL/6 mice received by

intravenous retro-orbital injection, 10^5 tumor cells incubated with medium or transfected with CXCR4-1 shRNA or control shRNA expressing plasmids. On day 21, mice were sacrificed by cervical dislocation, and the pulmonary metastatic nodules were counted using a dissecting microscope (M80; Leica Microsystems GmbH, Wetzlar, Germany).

Intravenous injection of jetPEI-CXCR4 shRNA. jetPEI™ is composed of linear polyethylenimine (Polyplus-Transfection SA, Illkirch, France) which ensures effective and repro-

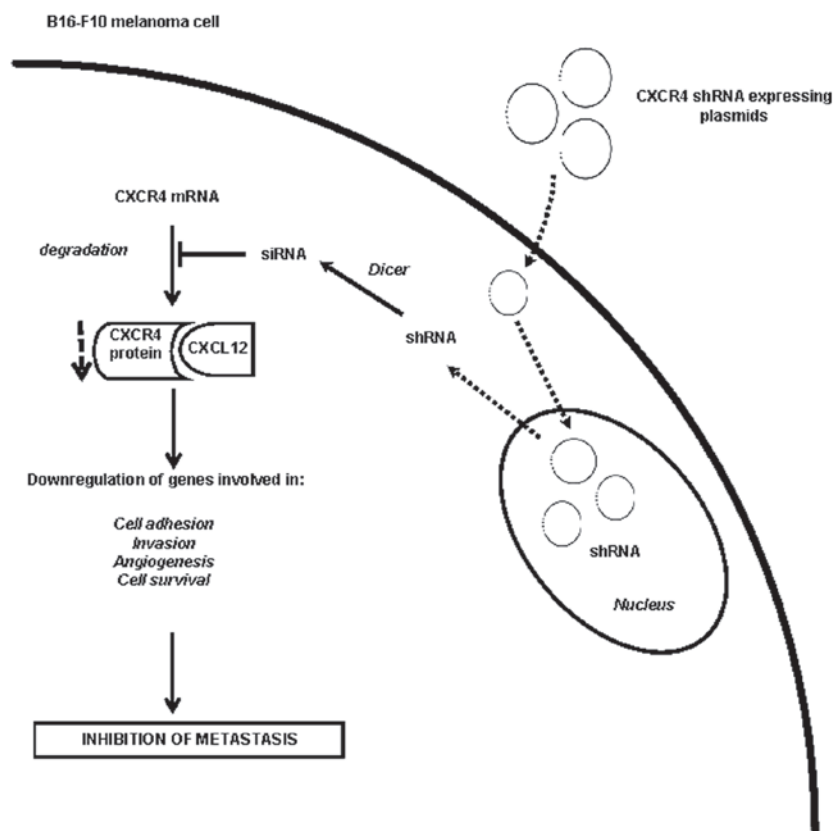


Figure 5. Overview of proposed molecular mechanisms involved in the reduction of pulmonary metastasis following CXCR4 knockdown with CXCR4-shRNA expressing plasmids. CXCR4, CXC motif chemokine receptor 4; CXCL12, CXC motif chemokine 12; shRNA, short hairpin RNA; siRNA, small interfering RNA.

ducible oligonucleotide transfection into mammalian cells with low toxicity. This reagent was a gift from Dr Catherine David (Biotik@ Biotechnologia, São Paulo, Brazil). C57BL/6 mice were inoculated with B16-F10 melanoma cells by retro-orbital injections (10^5 cells/animal). The mice were randomly separated into groups of 10 animals. A total of 10 μ g plasmid psiSTRIKE (control shRNA and CXCR4 shRNA) was diluted in 10% glucose solution at a 1:1 ratio and then mixed with jetPEI in the ratio of 1:1 and incubated at room temperature for 20 min. The jetPEI-CXCR4 shRNA was then injected retro-orbitally into the C57BL/6 mice, 12 h following the inoculation with B16-F10 cells. The mice were sacrificed 21 days following the injection of the tumor cells, and the pulmonary nodules were counted using a dissecting microscope (M80).

Statistical analysis. One-way analysis of variance was used to analyze statistical significance between the groups. Data are presented as the mean \pm standard deviation. Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

CXCR4 shRNA-expressing plasmids inhibit the expression of CXCR4 mRNA and protein. The ability of the two CXCR4-specific shRNAs expressing plasmid to downregulate

CXCR4 expression *in vitro* was investigated. B16-F10 melanoma cells were transfected for 24 and 48 h with the plasmid-based CXCR4-1 shRNA, CXCR4-2 shRNA or control shRNA in the presence of Lipofectamine 2000. Following *in vitro* transfection, CXCR-4 knockdown was measured by RT-PCR and western blot analysis. The results indicated that the CXCR4-1 shRNA significantly inhibited the expression of CXCR4 mRNA (80%, $P < 0.001$; Fig. 1) and CXCR4 protein (68%, $P < 0.001$; Fig. 2) following 48 h *in vitro* transfection of tumor cells. Transfection with CXCR4-2 shRNA did not result in a reduction in the mRNA or protein expression of CXCR4 (data not shown). Therefore, CXCR4-1 shRNA was used in all further experiments.

***In vitro* transfection of B16-F10 melanoma cells with CXCR4 shRNA inhibits pulmonary metastasis.** To investigate the effect of reducing CXCR4 expression on the metastatic potential of B16-F10 melanoma cells, the CXCR4-1 shRNA-transfected tumor cells were injected intravenously into mice. Fig. 3 indicates that the pulmonary metastatic nodules were significantly reduced ($\sim 85\%$; $P < 0.001$) in mice inoculated with CXCR4-1 shRNA-transfected B16-F10 melanoma cells compared with the control group.

Intravenous injection of jetPEI-CXCR4-1 shRNA inhibits pulmonary metastasis. The jetPEI-CXCR4-1 shRNA was injected retro-orbitally at 12 h following the inoculation of B16-F10 melanoma cells. Fig. 4 indicates that the

jetPEI-CXCR4-1 shRNA was able to significantly reduce (~50%; $P < 0.01$) the number of pulmonary metastatic nodules compared with the control group.

Discussion

The current study was conducted using B16-F10 melanoma cells, a variant cell line of B16 melanoma, which is highly metastatic to the lungs when injected intravenously into C57BL/6 mice (14). The intravenous injection of B16-F10 melanoma cells via the lateral tail vein is the most commonly used method of administration. However, this method has a high rate of failure and may result in distress due to the animals being placed under a heat lamp to promote peripheral vasodilatation (23). In the present study, retro-orbital injection was observed to be a reliable method for intravascular delivery of B16-F10 melanoma cells compared with injection via the tail vein. This model of metastasis has advantages, including the fact that the metastatic nodules are clearly observable in the lungs due to their high melanin content, and the ease with which the tumor cells may be grown in culture. Furthermore, the B16 murine melanoma is considered a good model of metastasis as it arose spontaneously, and it has been reported that this model accurately recapitulates clinical metastatic melanoma (15). In addition, B16-F10 melanoma cells constitutively express CXCR4 and it is possible to specifically inhibit this chemokine receptor expression through RNAi technology in order to elucidate the possible roles of CXCR4 in metastasis.

The B16-F10 melanoma cells transfected *in vitro* with CXCR4 shRNA were demonstrated to be effective in inhibiting pulmonary metastasis when intravenously injected into the mice, suggesting that CXCR4 serves a role in this model of metastasis. The next step was to investigate the effect of CXCR4 knockdown *in vivo* on the metastatic potential of B16-F10 melanoma cells. The instability and inadequate biodistribution of RNAi are key challenges to its clinical use, however nanoparticle formulations have been used to increase the efficacy of RNAi *in vivo*. Whilst numerous studies have been conducted to optimize nanoparticle gene delivery formulations *in vitro*, relatively few studies have attempted to deliver genes to experimental tumors *in vivo* (24,25). The current study used the polycation-based nanoparticle (jetPEI) to investigate the effect of CXCR4-1 knockdown *in vivo* on the metastatic potential of B16-F10 melanoma cells. This demonstrated that the retro-orbital injection of jetPEI-CXCR4-shRNA significantly reduced the number of pulmonary metastatic nodules. Fidler (14) demonstrated that tumor cells are observed in the lungs of mice shortly following intravenous injection. Furthermore, it was observed that the jetPEI-RNAi nanoparticles are predominantly located in the lung when intravenously injected in mice (26). This suggests that the results of the present study are likely to be due to CXCR4 knockdown *in vivo* by the jetPEI-CXCR4-shRNA nanoparticles present in the lungs.

The role of the CXCR4/CXCL12 axis in cancer has been extensively investigated. It was observed that CXCR4 is over-expressed in greater than 23 types of human cancer, including melanoma, and contributes to cell proliferation, cell survival, invasion and angiogenesis (3). Based on the results of the current study, the effect of CXCR4 shRNA expressing plasmids

may be explained as presented in Fig. 5. Briefly, CXCR4 shRNA expression plasmids were used to transfect B16-F10 melanoma cells *in vitro* or *in vivo* with jetPEI-CXCR4-1 shRNA. Following the uptake of CXCR4 shRNA expression plasmids by the tumor cells, CXCR4 shRNA is transcribed in the nucleus, exported to the cytoplasm and processed by dicer to generate CXCR4 siRNA, which induces the specific degradation of CXCR4 mRNA. Therefore, the level of CXCR4 protein is reduced with subsequent downregulation of genes involved in cell survival, cell adhesion, invasion and angiogenesis, resulting in the inhibition of pulmonary metastasis.

The present study demonstrated that CXCR4 serves a role in the formation and progression of pulmonary metastatic nodules in the experimental metastasis model using B16-F10 melanoma cells. However, further studies are required to elucidate the molecular mechanisms involved in this phenomenon. Currently there is interest in the discovery of antagonists for the therapeutic targeting of CXCR4 expression. Considering the results of the current study and the fact that CXCR4 is highly conserved between humans and mice (27), this experimental model of metastasis with B16-F10 melanoma cells may aid in the discovery of CXCR4 antagonists with clinical implications.

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