Chemokine receptor 4 gene silencing blocks neuroblastoma metastasis *in vitro*

Xin Chen[1], Yongjie Zhu[1], Lulu Han[2], Hongting Lu[1], Xiwei Hao[1], Qian Dong[1]

1 Department of Pediatric Surgery, the Affiliated Hospital of Qingdao University, Qingdao, Shandong Province, China
2 Operating Room, the Affiliated Hospital of Qingdao University, Qingdao, Shandong Province, China

Corresponding author:
Qian Dong, Department of Pediatric Surgery, the Affiliated Hospital of Qingdao University, Qingdao 266003, Shandong Province, China, dong.qian@sohu.com.

Abstract

This study investigated the effects of small interfering RNA (siRNA)-mediated silencing of chemokine receptor 4 (CXCR4) on the invasion capacity of human neuroblastoma cell line SH-SY5Y *in vitro*. Three siRNAs targeting *CXCR4* were chemically synthesized and individually transfected into SH-SY5Y cells. Expression of *CXCR4* mRNA and protein was significantly suppressed in transfected cells by all three sequence-specific siRNAs compared with control groups. Furthermore, the invasion capacity of SH-SY5Y cells was significantly decreased following transfection with *CXCR4*-specific siRNA compared with the control groups. These data demonstrate that down-regulation of *CXCR4* can inhibit *in vitro* invasion of neuroblastoma.

Key Words: nerve regeneration; chemokine receptor 4; small interfering RNA; neuroblastoma; invasion; Transwell chamber; liposome; NSFC grant; neural regeneration

Chen X, Zhu YJ, Han LL, Lu HT, Hao XW, Dong Q. Chemokine receptor 4 gene silencing blocks neuroblastoma metastasis in vitro. Neural Regen Res. 2014;9(10):1063-1067.

Introduction

Neuroblastoma is one of the most common solid tumors in children. It is highly malignant and metastasis occurs even in the early stages of the disease. Lung and brain metastases are very common and bone marrow metastases occur in 60% of cases; these are the main causes of death in patients with neuroblastoma (Ara and DeClerck, 2006). A theoretical model has been proposed in which tumor cells form organ-specific metastasis through the specific interaction of chemokine receptor 4 (CXCR4) and its ligand, CXCL12 (Muller et al., 2001). CXCR4 participates in tumor occurrence, growth and metastasis, and CXCR4 expression is up-regulated during neuroblastoma metastasis (Hao et al., 2005). In this study, RNA interference technology was used to investigate the effect of silenced *CXCR4* on the metastatic capacity of the human neuroblastoma cell line SH-SY5Y *in vitro*.

Materials and methods

Preparation of recombinant *CXCR4* siRNA plasmids

Three *CXCR4* sequence-specific siRNAs incorporating *Bam*HI and *Hin*dIII restriction enzyme sites for cloning purposes were designed and synthesized according to the human *CXCR4* cDNA sequence (GenBank accession No. NM-003467; **Table 1**). Formation of double-stranded DNA by annealing of siRNAs was confirmed by agarose gel electrophoresis. The pSilencer™ neo expression vector was digested with *Bam*HI and *Hin*dIII, and the resulting 4.5-kb fragments were separated by agarose gel electrophoresis. The siRNA fragments were ligated into the vector using T4 DNA ligase and then transformed into competent XL1-blue Escherichia coli by heat shock. Cells were then cultured on Lysogeny broth agar containing ampicillin to screen for monoclonal colonies. DNA was extracted by alkaline lysis and clones containing the correct insert were identified by diagnostic restriction enzyme digestion.

Culture and recombinant plasmid transfection of SH-SY5Y neuroblastoma cells

One day prior to transfection, SH-SY5Y cells were treated with 0.25% pancreatin, diluted with complete medium (serum supplemented) and inoculated into six-well plates (1×10^6 cells/well). Cells were cultured to 60–80% confluence, washed with serum-free Dulbecco's modified Eagle's medium (DMEM) three times and then 1.5 mL Opti-MEM (Gibco, Cat. No. 31985) serum-free medium was added to each well. Cells were transfected in the following groups and incubated for 30 minutes: (1) siRNA groups (1, 2 and 3): mixture containing 25 μg Silencer/siRNA plasmid (1, 2 or 3) (final concentration of siRNA: 50 nmol/L), 240 μL Opti-MEM serum-free medium and 10 μL Lipofectamine 2000 (Invitrogen, Cat. # 11668); (2) blank control group: untreated neuroblastoma SH-SY5Y cells; (3) empty vector control group: empty plasmid + liposome control group, mixture containing 25 μg empty plasmid, 240 μL Opti-MEM serum-free medium and 10 μL Lipofectamine 2000. After 6 hours of culture, the supernatant was removed and replaced with complete me-

Table 1 *CXCR4* siRNA sequences

Target	Sequence (5′–3′)
CXCR4-siR1	Forward: GAT CCG CAT GAC GGA CAA GTA CAG TTC AAG AGA CTG TAC TTG TCC GTC ATG CTT TTT TGG AAA Reverse: AGC TTT TCC AAA AAA GCA TGA CGG ACA AGT ACA GTC TCT TGA ACT GTA CTT GTC CGT CAT GCG
CXCR4-siR2	Forward: GAT CCG TGG ATT TCC ATC ACC GAG TTC AAG AGA CTC GGT GAT GGA AAT CCA CTT TTT TGG AAA Reverse: AGC TTT TCC AAA AAA GTG ATT TCA T CAC CGA GTC TCT TGA ACT CGG TGA TGG AAA TCC ACG
CXCR4-siR3	Forward: ATC CGC TGA AGC ATG ACG GAC A TTC AAG AGA TGT CCG TCA TGC TTC TCA GTT TTT TGG AAA Reverse: GCT TTT CCA AAA AAC TGA GAA GCA TGA CGG ACA TCT CTT GAA TGT CCG TCA TGC TTC TCA GCG

CXCR4: chemokine receptor 4.

Table 2 RT-PCR primers

Gene	Sequence (5′–3′)	Product size (bp)
CXCR4	Forward: CTT ACT ACA TTG GGA TCA GC Reverse: AGT CCT ACC ACG AGA CAT AC	546
β-Actin	Forward: CGT GAC ATT AAG GAG AAG CTG Reverse: CTA GAA GCA TTT GCG GTG GAC	500

CXCR4: chemokine receptor 4.

dium and culture was continued for a further 72 hours. Efficient transfection was confirmed by visualization of green fluorescent protein using fluorescence microscopy.

Semi-quantitative RT-PCR analysis of *CXCR4* mRNA levels in siRNA-transfected SH-SY5Y cells

Cells were harvested 72 hours after transfection and total RNA was extracted using the Trizol one-step method. The RNA concentration was estimated by ultraviolet spectrophotometry, and RNA integrity was assessed by 1% agarose gel electrophoresis. Forward and reverse PCR primers (synthesized by Sangon Biotech, Shanghai, China) were designed using Primer Premier 5.0 software for the amplification of *CXCR4* (546-bp product) and *β-actin* (500-bp product) (primer sequences are listed in **Table 2**).

The following conditions were used for PCR amplification: 94°C for 3 minutes; 30 cycles of 94°C for 40 seconds, 56°C for 30 seconds and 72°C for 60 seconds, with a final extension step at 72°C for 10 minutes. The correct sizes of amplification products were confirmed by 1% agarose gel electrophoresis. The integrated absorbance of the bands was analyzed using a gel imaging system and the ratio of *CXCR4/β-actin* was calculated to represent the relative level of *CXCR4* mRNA.

Immunocytochemical detection of CXCR4 protein in siRNA-transfected SH-SY5Y cells

Cells in the logarithmic growth phase were harvested 72
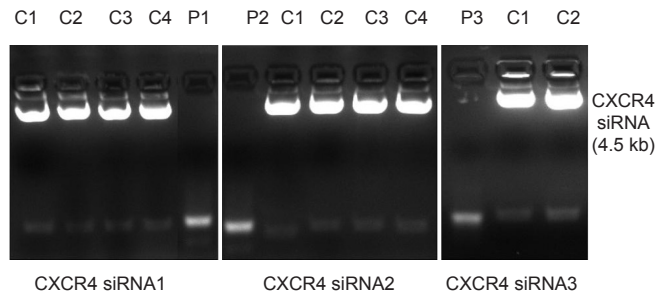
Figure 1 *CXCR4* siRNA recombinant plasmid construction and verification.
Diagnostic restriction enzyme digestion indicated correct incorporation of target siRNA fragments (4.5 kb, arrow) in each clone (siRNA 1, 2 and 3). P1–3: Product 1–3; C1–4: CXCR4 siRNAs.

hours after transfection and mounted onto microscope slides (polylysine treated and high-pressure sterilized), rinsed with PBS and fixed in acetone at –4°C for 30 minutes. Immunocytochemical staining was performed using the PV-6000 method. Five randomly selected fields were analyzed using ImagesPlus software and the average gray value was calculated.

Western blot assay of CXCR4 protein levels in siRNA-transfected SH-SY5Y cells

Cells were harvested 72 hours after siRNA transfection and lysed by addition of radioimmune precipitation assay lysis buffer for extraction of total cellular proteins. Protein concentrations were measured using a bicinchoninic acid kit. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, blocked, and incubated with rabbit-anti-human CXCR4 monoclonal antibody (1:200) overnight at 4°C. Membranes were incubated with a horseradish peroxidase-conjugated secondary detection antibody (1:500) for 2 hours. Proteins were visualized by enhanced chemiluminescence and analyzed using a gel imaging system to scan the band gray value. CXCR4 (43 kDa) protein was quantified with reference to the β-tubulin (55 kDa) internal control.

Chemotaxis assay to assess metastatic capacity of SH-SY5Y neuroblastoma cells

Transwell chamber polycarbonate membranes were layered with extracellular matrix matrigel (approximately 40 μg/well), incubated at 37°C for 5 hours to enable polymerization and dried at room temperature overnight. Transfected cells (100 μL; cell density, 2.5×10^5/mL) were added to the lower transwell chamber. High-glucose DMEM (600 μL) containing 10% fetal bovine serum and stromal cell-derived factor 1 (CXCL12, 100 ng/mL) was added to the upper chamber. Transwell chemotaxis plates were incubated in 5% CO_2 at 37°C for 48 hours. Cells on the polycarbonate membrane in the upper chamber were then removed, fixed with 4% paraformaldehyde for 30 minutes and stained with hematoxylin. The total number of cells that had migrated to the upper chamber was calculated by examination under an
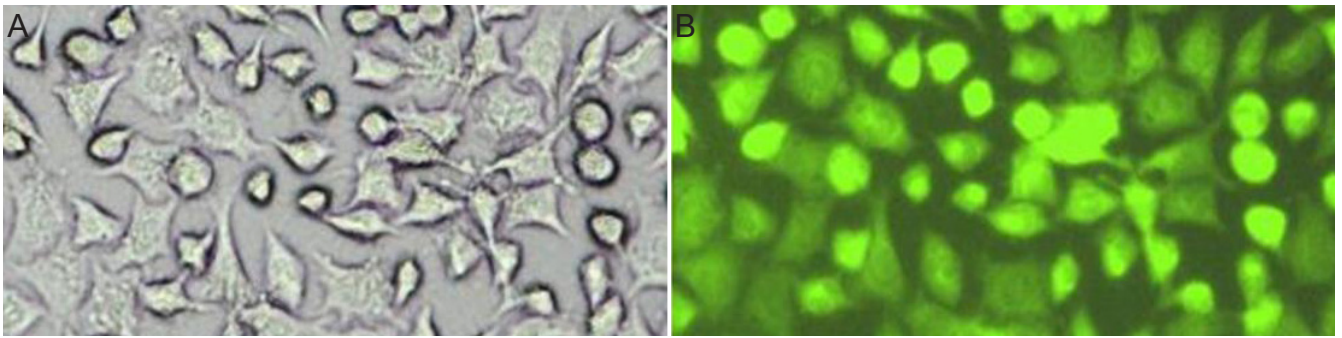
Figure 2 Efficiency of SH-SY5Y transfection with *CXCR4* siRNA plasmid.
Confirmation of efficient transfection of SH-SY5Y cells by visualization of green fluorescent protein reporter gene co-expression 72 hours after transfection. (A) Light microscopy image, × 100; (B) fluorescence microscopy image, wavelength 488 nm, × 100.
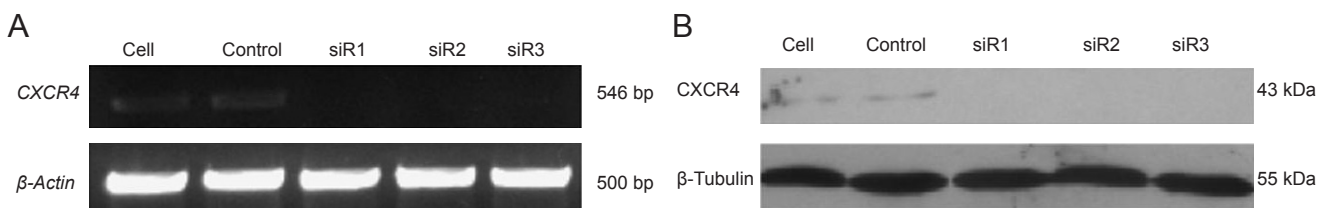

Figure 3 Detection of *CXCR4* mRNA (A) and protein (B) in siRNA-transfected SH-SY5Y cells by RT-PCR and western blot assay.
(A)The relative absorbance of CXCR4 mRNA in the test groups (siR1, siR2 and siR3) was significantly lower than that in the empty vector control group and blank control group. (B) Compared with the empty vector control group and blank control group, the expression of CXCR4 protein was significantly decreased following transfection with CXCR4-specific siRNAs.
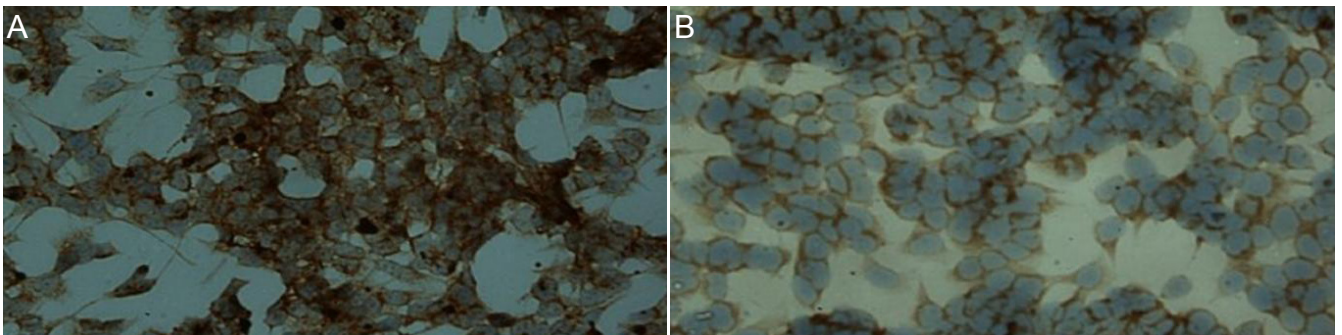

Figure 4 Immunocytochemical detection of CXCR4 protein in siRNA-transfected SH-SY5Y cells (light microscopy).
CXCR4 protein in cytoplasm and cell membranes was detected as brown/yellow particles. (A, × 200) CXCR4 in the negative control group is strongly expressed on the cell membrane and in the cytoplasm. (B, × 200) After transfection, CXCR4 in the siR1 transfection group is weakly expressed on the cell membrane and in the cytoplasm.

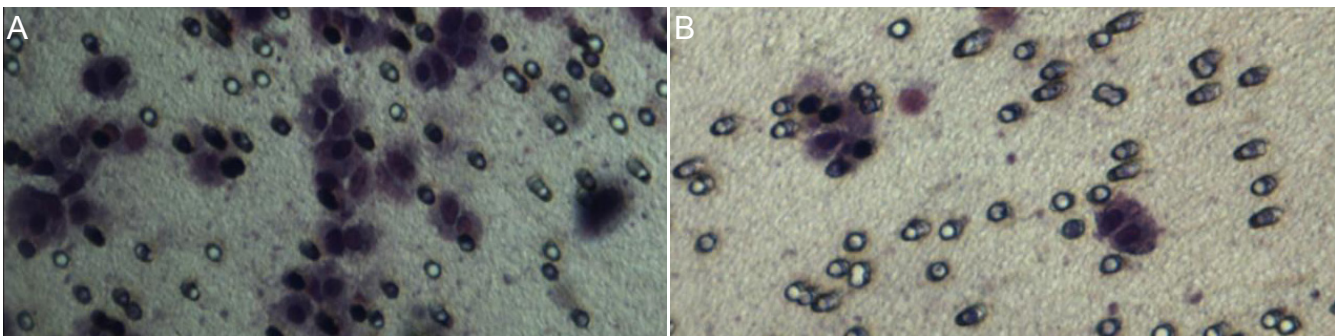**Figure 5 Chemotaxis assay of SH-SY5Y neuroblastoma cells (inverted microscopy, × 100).**
The extracellular matrix invasion capacity of each group was assessed as the number of cells passing from the lower to the upper chamber in chemotaxis assays. Compared with the empty vector control group and blank control group (A), cell invasion capacity was significantly decreased following transfection with CXCR4-specific siRNA (B).

inverted microscope. The mean number of cells in five fields of view was counted in each well.

Statistical analysis

Data were expressed as mean ± SD. Results from different groups were statistically assessed with *t*-tests using SPSS 10.0 software. A value of *P* < 0.05 was considered statistically significant.

Results

CXCR4 siRNA recombinant plasmid construction and verification

Restriction enzyme digestion indicated correct incorporation of target siRNA fragments in each clone (**Figure 1**). This was confirmed by sequence analysis (performed by Tianjin Saier Purcell Biotechnology in China).

CXCR4 siRNA plasmid transfection efficiency of neuroblastoma SH-SY5Y cells

Efficient transfection was confirmed by co-expression of a green fluorescent protein reporter gene visualized by fluorescence microscopy. Transfection efficiency was 70% (**Figure 2**).

CXCR4 mRNA levels in siRNA-transfected SH-SY5Y cells

The relative absorbance values of *CXCR4* RT-PCR assays in the test groups (siR1, siR2 and siR3) were 0.32 ± 0.09, 0.35 ± 0.13 and 0.33 ± 0.11, respectively. These values were significantly lower than 0.62 ± 0.10 for the empty vector control group and 0.58 ± 0.13 for the blank control group (*P* < 0. 05; **Figure 3A**).

Immunocytochemical detection of CXCR4 protein in siRNA-transfected SH-SY5Y cells

CXCR4 protein in cytoplasm and cell membranes was detected as brown/yellow particles. The mean absorbance values in each group were: blank control, 92.20 ± 3.89; empty vector control, 94.20 ± 4.19; siR1, 75.98 ± 4.81; siR2, 75.52 ± 3.95; siR3 76.35 ± 6.51. Compared with the empty vector control and blank control groups, the expression of CXCR4 protein was significantly decreased following transfection with CXCR4-specific siRNAs (*P* < 0.01; **Figure 4**).

CXCR4 protein levels in siRNA-transfected SH-SY5Y cells

The relative gray values of CXCR4 protein analyzed by western blot assay were: blank control, 0.4832 ± 0.0012; empty vector control, 0.4231 ± 0.0023; siR1, 0.1103 ± 0.0023; siR2, 0.1203 ± 0.0015; siR3 0.1308 ± 0.0018. Compared with the empty vector control and blank control groups, the level of CXCR4 protein was significantly decreased following transfection with CXCR4-specific siRNAs (*P* < 0.01; **Figure 3B**).

Chemotaxis assay of metastatic capacity of SH-SY5Y neuroblastoma cells

Extracellular matrix invasion capacity of each group was assessed in chemotaxis assays as the number of cells passing from the lower to the upper chamber. The results for each group were: blank control, 53.11 ± 3.72; empty vector control, 49.53 ± 2.78; siR1, 25.48 ± 2.81; siR2, 30.89 ± 2.77; siR3 18.83 ± 1.79. Compared with the empty vector control and blank control groups, cell invasion capacity was significantly decreased following transfection with CXCR4-specific siRNAs (*P* < 0.05; **Figure 5**).

Discussion

Chemokines, originally named for their ability to induce leukocyte chemotaxis, are a superfamily of inducible pro-inflammatory cytokines. They are single-stranded proteins (8–10 kDa) and are produced by a variety of cells. CXCR4 (chemokine receptor 4) is a highly conserved G protein-coupled seven-transmembrane receptor consisting of 352 amino acids. In this study, RNA interference technology was adopted to investigate the effect of silenced CXCR4 expression on the *in vitro* invasion capacity of the human neuroblastoma cell line SH-SY5Y.

The interaction between chemokine CXCL12 and its specific receptor, CXCR4, known as the CXCR4-CXCL12 axis, involves a specific high-affinity interaction between these two closely related molecules for the transfer of intercellular information and induction of cell migration. Chemokines play an important role in tumor-initiated organ metastasis with "homing theory" proposed as the main mechanism underlying this process (Cui et al., 1999). A theoretical model for organ-specific tumor metastasis is based on the binding of a specific tumor-associated chemokine receptor and its ligand (Jiang and Wu, 2007). Tumor models in animals have shown that expression of CXCL12 and CXCR4 was upregulated in neuroblastoma metastases (Hao et al., 2005). Therefore, investigation of the CXCR4-CXCL12 axis in tumor invasion and metastasis represents a new focus for research into the mechanism of metastasis in neuroblastoma.

The CXCR4-CXCL12 axis induces the chemotactic migration of tumor cells and plays an important role in driving tumor invasion and organ-specific metastasis. This involves rearrangement of the intracellular skeleton and enhancement of target cell adhesion capacity. Furthermore, the CXCR4-CXCL12 axis is involved in the occurrence, development and prognosis of malignant tumors through the induction and guidance of leukocyte infiltration into tumor tissues, regulation of angiogenesis and direct activation and regulation of tumor cell functions (Vandercappellen et al., 2008; Yoshitake et al., 2008). CXCR4-CXCL12-mediated tumor cell proliferation and invasion occur through a signal transduction mechanism. Binding of CXCR4 and CXCL12 leads to G-protein structural changes and activation of the mitogen-activated protein kinase, extracellular signal-regulated kinase 1/2 and SAPK/JNK pathways. MEK-1 is the upstream signal transduction pathway that activates extracellular signal-regulated kinase 1/2, mitogen-activated protein kinase and phosphoinositide-3 kinase pathways resulting in nuclear factor- B activation. Activated CXCR4 also activates the Akt pathway, and extracellular signal-regulated kinase 1/2 and Akt activation leads to resistance to cell apoptosis and promotes proliferation (Balkwill, 2004). Binding of CXCL12 to CXCR4 also promotes hematopoiesis through

a multi-signaling pathway that induces vascular endothelial growth factor secretion, resulting in angiogenesis and tumor cell growth and invasion (Salvucci et al., 2002). Following tumor cell stimulation by CXCL12, high-level F-actin polymerization and redistribution to the front of the cell with significant pseudopod formation is visible. This is necessary for malignant cell invasion and metastasis.

In this study, efficient transfection of the SH-SY5Y human neuroblastoma cell line with siRNA-encoding plasmids carrying a green fluorescent protein reporter gene was confirmed by fluorescence microscopy visualization of green fluorescent protein co-expression. Expression of *CXCR4* mRNA was detected by semi-quantitative RT-PCR 72 hours after transfection and CXCR4 protein was detected by immunocytochemistry and western blot assays. The chemotactic invasion capacity of transfected cells was assessed in extracellular matrix gel transwell chemotaxis assays. Data analysis revealed significantly decreased levels of *CXCR4* mRNA and protein as well as reduced cell invasion and metastasis capacity following transfection with *CXCR4*-specific siRNAs compared with the empty vector control and blank control groups. Therefore, these data confirm siRNA-mediated down-regulation of *CXCR4* expression and indicate that this molecule is involved in metastasis of neuroblastoma. Furthermore, it was observed that the high *in vitro* invasion capacity of the SH-SY5Y neuroblastoma cell line correlates with high levels of *CXCR4*, thus implicating the CXCR4-CXCL12 axis in neuroblastoma invasion and metastasis. CXCL12 is highly expressed in bone marrow, a common site for neuroblastoma metastasis. Therefore, we speculate that the CXCR4-CXCL12 axis is pivotal to the development of bone marrow-specific metastasis in neuroblastoma. This hypothesis requires further investigation and may provide new targets for the treatment of neuroblastoma.

Although the results of this study demonstrate a relationship between CXCR4 expression and the invasion capacity of SH-SY5Y cells *in vitro*, it should be noted that the relationship between CXCR4 expression and invasion and metastasis in neuroblastoma is currently in dispute. Furthermore, non-functional CXCR4 expression has been identified in the tissues of patients with neuroblastoma (Airoldi et al., 2006). Results obtained by Meier et al. (2007) are in conflict with those obtained in this study. However, this may be attributed to differences in cell lines investigated.

In conclusion, specific siRNA-mediated down-regulation of *CXCR4* expression results in reduced invasion capacity in SH-SY5Y cells. However, the complex network of intracellular chemokines and the signaling pathways involved remain to be identified. Such information will provide a greater understanding of the mechanism of metastasis in neuroblastoma and inform the selection of novel targets for therapy.

References

Airoldi I, Raffaghello L, Piovan E, Cocco C, Carlini B, Amadori A, Corrias MV, Pistoia V (2006) CXCL12 does not attract CXCR4⁺ human metastatic neuroblastoma cells: clinical implications. Clin Cancer Res 12:77-82.

Ara T, DeClerck YA (2006) Mechanisms of invasion and metastasis in human neuroblastoma. Cancer Metast Rev 25:645-657.

Balkwill F (2004) The significance of cancer cell expression of the chemokine receptor CXCR4. In: Semin Cancer Biol 171-179.

Cui J, Wahl RL, Shen T, Fisher SJ, Recker E, Ginsburg D, Long MW (1999) Bone marrow cell trafficking following intravenous administration. Brit J Haematol 107:895-902.

Hao X, Dong Q, Lu H, Lv Z, Sun L, Jiang B (2005) Establishment of neuroblastoma's model in mice. Zhonghua Xiaoer Waike Zazhi 26: 372-375.

Jiang Y, Wu X (2007) Correlations of chemokine CXCL12 and its receptor to tumor metastasis. Aizheng 26:220-224.

Meier R, Mühlethaler-Mottet A, Flahaut M, Coulon A, Fusco C, Louache F, Auderset K, Bourloud KB, Daudigeos E, Ruegg C (2007) The chemokine receptor CXCR4 strongly promotes neuroblastoma primary tumour and metastatic growth, but not invasion. PLoS One 2:e1016.

Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN (2001) Involvement of chemokine receptors in breast cancer metastasis. Nature 410:50-56.

Salvucci O, Yao L, Villalba S, Sajewicz A, Pittaluga S, Tosato G (2002) Regulation of endothelial cell branching morphogenesis by endogenous chemokine stromal-derived factor-1. Blood 99:2703-2711.

Vandercappellen J, Van Damme J, Struyf S (2008) The role of CXC chemokines and their receptors in cancer. Cancer Lett 267:226-244.

Yoshitake N, Fukui H, Yamagishi H, Sekikawa A, Fujii S, Tomita S, Ichikawa K, Imura J, Hiraishi H, Fujimori T (2008) Expression of SDF-1 alpha and nuclear CXCR4 predicts lymph node metastasis in colorectal cancer. Brit J Cancer 98:1682-1689.