

## RESEARCH ARTICLE

# Hiwi Knockdown Inhibits the Growth of Lung Cancer in Nude Mice

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### Abstract

Hiwi, a human homologue of the Piwi family, plays an important role in stem cell self-renewal and is overexpressed in various human tumors. This study aimed to determine whether an RNA interference-based strategy to suppress Hiwi expression could inhibit tumor growth in a xenograft mouse model. A rare population of SSC<sup>lo</sup>Alde<sup>br</sup> cells was isolated and identified as lung cancer stem cells in our previous study. Plasmids containing U6 promoter-driven shRNAs against Hiwi or control plasmids were successfully established. The xenograft tumor model was generated by subcutaneously inoculating with lung cancer stem cell SSC<sup>lo</sup>Alde<sup>br</sup> cells. After the tumor size reached about 8 mm in diameter, shRNA plasmids were injected into the mice via the tail vein three times a week for two weeks, then xenograft tumor growth was assessed. In nude mice, intravenously delivery of Hiwi shRNA plasmids significantly inhibited tumor growth compared to treatment with control scrambled shRNA plasmids or the vehicle PBS. No mice died during the experiment and no adverse events were observed in mice administered the plasmids. Moreover, delivery of Hiwi shRNA plasmids resulted in a significant suppressed expression of Hiwi and ALDH-1 in xenograft tumor samples, based on immunohistochemical analysis. Thus, shRNA-mediated Hiwi gene silencing in lung cancer stem cells by an effective *in vivo* gene delivery strategy appeared to be an effective therapeutic approach for lung cancer, and may provide some useful clues for RNAi gene therapy in solid cancers.

**Keywords:** Hiwi gene - RNA interference - shRNA - lung cancer stem cell - SSC<sup>lo</sup>Alde<sup>br</sup> cells

*Asian Pacific J Cancer Prev*, 14 (2), 1067-1072

### Introduction

Lung cancer is a lethal disease and is the leading cause of cancer-related death worldwide (Siegel et al., 2012). There are two major types of lung cancer: non-small cell lung cancer (about 85% of all lung cancers) and small-cell lung cancer (about 15%) (Takei et al., 2004). Non-small-cell lung cancer consists of three major histologic subtypes: squamous-cell carcinoma, adenocarcinoma, and large-cell lung cancer (Gurzov et al., 2006). Although some advances had been made in early detection and standard treatment, non-small-cell lung cancer is often diagnosed at the advanced stages due to lack of symptoms or nonspecific symptoms, leading to a poor prognosis (Zamore et al., 2000). Studies showed that improvements in standard chemotherapy for lung cancer have been mostly palliative with a one-year survival of only 35% (Zamore et al., 2000). Consequently, there is a pressing need for the development of new therapeutic methods to improve the survival of lung cancer patients.

RNA interference (RNAi) is a regulatory mechanism of most eukaryotic cells that uses small double stranded RNA (dsRNA) molecules to direct homology-dependent control

of gene activity (Zamore et al., 2000). Recently, some breakthroughs had been made with use of RNAi for cancer therapeutics. Some studies showed that adenoviral or retroviral delivery of shRNAs targeting Hec1 significantly reduced the tumor growth in nude mice (Gurzov et al., 2006), atelocollagen-complexed siRNA that effectively silenced Vascular endothelial growth factor (VEGF) could inhibit angiogenesis and promote tumor regression *in vivo* (Takei et al., 2004), and transferrin receptor targeted cyclodextrin nanoparticles that delivered anti Ews-Fli1 siRNAs to human Ewing's tumors can effectively block the metastasis (Hu-Lieskovan et al., 2005). However, there are rare reports about RNA therapeutics for lung cancer.

Cancer stem cells (CSCs) are a rare group of undifferentiated tumorigenic cells which are considered to be the renewable source of tumor cells and a source of drug resistance leading to tumor recurrence, metastasis, and tumor progression (Clarke et al., 2006; Visvader et al., 2008). CSCs were firstly identified in human leukemia (Hu-Lieskovan et al., 2005), and subsequently in a great number of solid tumors including in lung cancers (Eramo et al., 2008). Recent studies indicate that CSCs which are self-renewing and differentiate into heterogeneous

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tumor cells, are responsible for the drug resistance and tumor relapse (Yu et al., 1995). Hiwi gene, a human homologue of the Piwi family which plays an important role in stem cell self-renewal (Cox et al., 1998; Hutvagner et al., 2008), had been shown to be highly expressed in a variety of human cancers (Liu et al., 2006; Grochola et al., 2008). Moreover, recent data suggested that Hiwi gene was required to maintain the stemness of hematopoietic stem cells (Sharma et al., 2001). Thus it was proposed that Hiwi gene may be a key regulator in maintenance of cancer stem cell populations as well (Taubert et al., 2007; Siddiqi et al., 2012).

In present study, we hypothesized that Hiwi knockdown in lung cancer stem cells might exhibit antitumor effects to lung cancer. To test the hypothesis, a lung cancer stem cell population (SSC<sup>lo</sup>Alde<sup>br</sup> cells) had been successfully isolated from the lung adenocarcinoma cell line SPC-A1 cells using flow cytometry in our previous study (Liang & Shi, 2012), a short hairpin RNA (shRNA) against Hiwi had also been successfully established as described previously (Liang et al., 2012), and then we investigated whether silencing of Hiwi in lung cancer stem cells can modulate tumor growth in nude mouse.

## Materials and Methods

### Cell culture

The SSC<sup>lo</sup>Alde<sup>br</sup> cells had been successfully isolated from the SPC-A1 cells and established as lung cancer stem cells in our previous study (Liang & Shi, 2012). The SSC<sup>lo</sup>Alde<sup>br</sup> cells were cultured in serum-free Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with B27 (1:50), 20 ng/ml recombinant human epidermal growth factor, 20 ng/ml recombinant basic fibroblast growth factor, 40 U/ml dexamethasone, 2 mmol/l glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, and 0.5 µg/ml hydrocortisone. Cells were cultured incubated in a humidified incubator at 37 °C under a 5% CO<sub>2</sub> atmosphere.

### Construction of Hiwi shRNA plasmids

A short hairpin RNA (shRNA) against Hiwi (pGenesil-2-Hiwi2263) and a negative control shRNA (pGenesil-2-control) were designed and chemically synthesized (Shanghai Sangon Biotech Co., Shanghai, China) for targeting the coding regions of the gene as described previously (Liang et al., 2012). The shRNA insert was created using restriction site enzyme digestion with BamH I at the 5' end and Hind III at the 3' end and then inserted into the blank expression plasmid pGenesil-2-U6-hygro (Wuhan Genesil Biotechnology Co., Ltd., Wuhan, China) to construct a recombinant plasmid vector that expressed the shRNAs. Then, the recombinant plasmid was validated by restriction enzymes digestion and sequencing.

### Mouse tumor model

Male specific pathogen-free BALB/c nude mice (6 weeks old and weighed 18-22g) were obtained from Harbin Medical University (Harbin, China). The mice were housed in the Animal Institute of Harbin Medical University in laminar flow cabinets under a specific

pathogen-free environment with access to food and water ad libitum. The animals were acclimatized for 1 week before use, and maintained throughout at standard conditions: 25 ± 2 °C temperature, 40%~60% relative humidity, and 12-hour light/12-hour dark cycle. After disinfecting the nude mice skin with 75% ethanol, 1×10<sup>5</sup> SSC<sup>lo</sup>Alde<sup>br</sup> cells were injected subcutaneously into the neck back using a sterile syringe. Mice were monitored to check for the appearance of signs of disease, such as subcutaneous tumors or weight loss due to potential tumor growth in internal sites. The growth of tumors was monitored throughout the experiment and tumor size was measured with calipers every 3 days. Tumor volume was determined as  $\pi l s^2/6$ , where  $l$  = long side and  $s$  = short side.

### Plasmid treatment

When tumor diameters reached about 8mm in size, mice were randomized into three groups (n = 6 for each): 1) pGenesil-2-Hiwi2263 group, which were injected with 4 µg of pGenesil-2-Hiwi2263 and 16 µg of polyethylenimine (PEI; Sigma Co., St. Louis, MO, USA) in a final volume of 200 µL sterile PBS through the tail vein; 2) pGenesil-2-control group, which were injected with 4 µg of pGenesil-2-control and 16 µg of PEI in a final volume of 200 µL sterile PBS through the tail vein; 3) PBS group, which were injected with 16 µg of PEI in a final volume of 200 µL sterile PBS through the tail vein. The plasmids were given thrice a week for two weeks (6 total injections). The tumor size was measured with calipers every week. The animal experiments were terminated at 28d, all nude mice were sacrificed by CO<sub>2</sub> asphyxiation. Mice were weighed, and tumors were excised and weighed. All animal experiments were approved by the Animal Care and Use Committee of our university and were carried out in compliance with the 'Guide for the Care and Use of Laboratory Animals' published by the National Institutes of Health.

### Immunohistochemistry assessment of Hiwi and ALDH1 expression

Tumor samples were subjected to immunohistochemical examination to determine the expression of Hiwi and ALDH1. Tissues were fixed in 4% paraformaldehyde at 4°C, embedded in paraffin, cut into 5µm sections, and transferred to silicon-coated slides, which were then stained with a mouse monoclonal antibody against Hiwi (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:30 or a mouse monoclonal antibody against ALDH1 (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:100. Visualization was performed using the 3, 3'-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, Burlingame, CA), followed by counterstaining with Mayer's hematoxylin (Merck, Darmstadt, Germany). The Hiwi or ALDH1 immunostained slides were evaluated on light microscopy using a total magnification of 400x and a 10x10 square grid placed in the ocular. The tumor cells were considered positive when they displayed a distinct cytoplasmic and nuclear reaction. The number of positive tumor cells was recorded after counting 500 tumor cells in consecutive high-power

fields in the most reactive areas on the slide. Cells with questionable nuclear staining were discounted. Necrotic or thick areas and severely overlapping tumor cells were avoided during evaluation. The Hiwi and ALDH1 labelling indexes (LI) were determined by counting 500 tumor cells, and were calculated as the percentage of positive labeled cells.

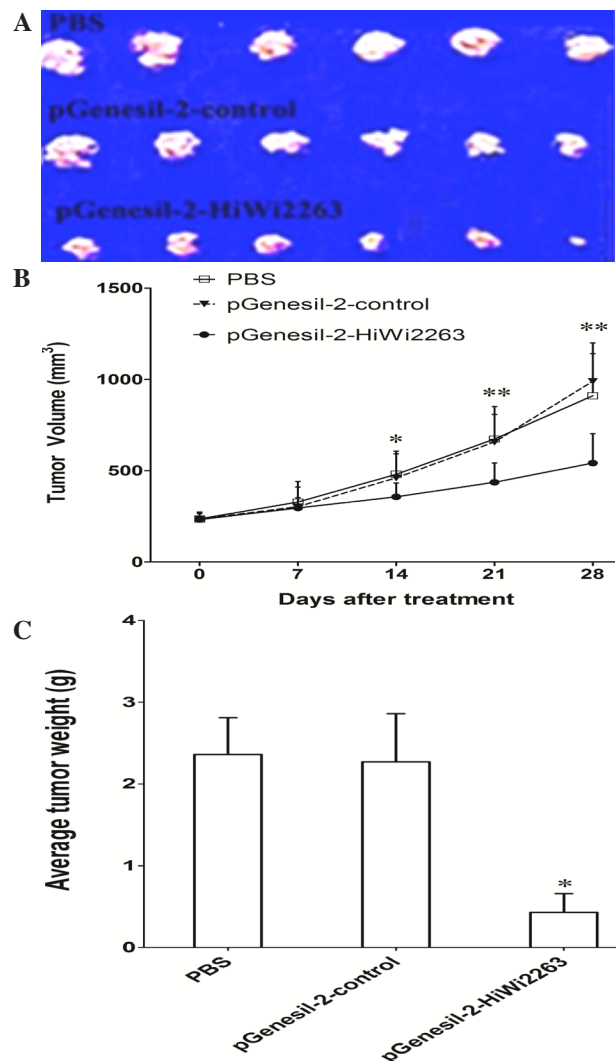
#### Statistical analysis

GraphPad Prism version 5.03 (GraphPad, San Diego, CA, USA) was used for all statistical analyses. All data are expressed as mean  $\pm$  SEM. Differences between groups were analyzed by a 2-tailed Student's paired t-test for single comparisons and by one-way ANOVA with LSD post-hoc test for multiple comparisons. Bonferroni's correction was used to adjust for multiple comparisons. A *P* value  $< 0.05$  was considered to be statistically significant.

## Results

#### Antitumor Activity of Hiwi shRNA In Vivo

A short hairpin RNA (shRNA) against Hiwi (pGenesil-2-Hiwi2263) and a negative control shRNA (pGenesil-2-control) were successfully established as described previously (Liang et al., 2012). To assess whether Hiwi knockdown could inhibit tumor growth in vivo, the nude mouse xenograft tumor models were established by subcutaneously inoculation of  $1 \times 10^5$  SSC<sup>lo</sup>Alde<sup>br</sup> cells. The tumors were allowed to reach 8 mm in diameter before administration of plasmids or vehicle. Mice with tumors were then randomized into three groups and treated with pGenesil-2-Hiwi2263 plasmid, pGenesil-2-control plasmid or the vehicle PBS, respectively. The regimen was carried out by bolus intravenous injection via the tail vein three times a week for six consecutive times. As shown in Figure 1, treatment with Hiwi shRNA plasmids statistically significantly reduced the growth of tumors in mice compared with treatment with the negative control shRNA plasmids or the vehicle PBS (Figure 1A and B). By the 14d, significant differences in tumor volumes began to be observed between pGenesil-2-Hiwi2263 group and pGenesil-2-control group,  $357.3 \pm 75.1$  mm<sup>3</sup> and  $460.8 \pm 132.5$  mm<sup>3</sup>, respectively (*P*  $< 0.05$ , Figure 1B). Similar differences were recorded between pGenesil-2-Hiwi2263 group and PBS group,  $357.3 \pm 75.1$  mm<sup>3</sup> and  $480.5 \pm 127.3$  mm<sup>3</sup>, respectively (*P*  $< 0.05$ , Figure 1B). At the termination of the experiment, tumors from vehicle PBS treated and the negative control shRNA plasmids mice grew to an average size of  $911.2 \pm 230.6$  mm<sup>3</sup> and  $990.5 \pm 210.7$  mm<sup>3</sup>, respectively; in contrast, tumors in mice treated with pGenesil-2-Hiwi2263 plasmids grow significantly less ( $542.2 \pm 161.1$  mm<sup>3</sup>, *P*  $< 0.01$ , Figure 1B). No significant differences were observed between the pGenesil-2-control group and the PBS group. Moreover, the average mass of tumor samples in Hiwi knockdown group is approximately 3/4-fold (by weight, *P*  $< 0.05$ ) decrease versus pGenesil-2-control group, and 3/4-fold (*P*  $< 0.05$ ) decrease versus PBS group (Figure 1C). No mice died during the experiment and there was no reduction in the body weight of mice treated with Hiwi shRNA or scrambled negative control



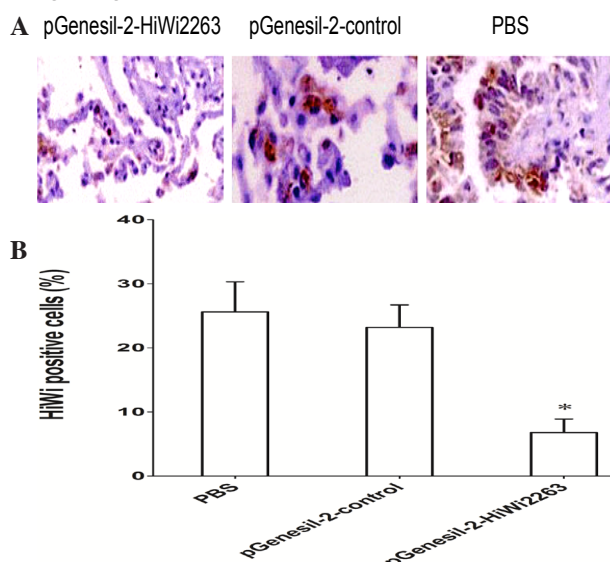
**Figure 1. Hiwi-specific Short Hairpin RNAs (shRNAs) Driven by U6 Promoters Inhibited the Growth of SSC<sup>lo</sup>Alde<sup>br</sup> Xenograft Tumors in Nude Mice.** SSC<sup>lo</sup>Alde<sup>br</sup> cells ( $1 \times 10^5$ ) were subcutaneously injected into BALB/c nude mice for a total of 0.1 mL. When tumor diameters reached about 8mm in size, mice were administered with pGenesil-2-Hiwi2263 plasmids, pGenesil-2-control plasmids, or the vehicle PBS via the tail vein. The plasmids were given three times a week for 14 days. The tumor diameters were measured with a caliper every week and the tumor volume was calculated as  $v = \pi l s^2 / 6$ , where *l* = long side and *s* = short side. The animal experiments were terminated at 28d, all nude mice were sacrificed by CO<sub>2</sub> asphyxiation. Mice were weighed, and tumors were excised and weighed. (A) Tumor samples collected at the termination of the experiment. (B) Tumor growth curve of the three groups. Data represent means  $\pm$  SEM of six samples. \**P*  $< 0.05$ ; \*\**P*  $< 0.01$ . (C) Total tumor weight (g) from each group of mice was calculated and shown. Data are means  $\pm$  SEM of six samples. \**P*  $< 0.05$

shRNA plasmids. We also found no obvious signs of adverse events in mice administered with the plasmids. Thus, treatment of Hiwi shRNA plasmids clearly inhibited the tumor growth of SSC<sup>lo</sup>Alde<sup>br</sup> cells in nude mice.

#### Decreased Expression of Hiwi, ALDH1 and Antitumor Activity

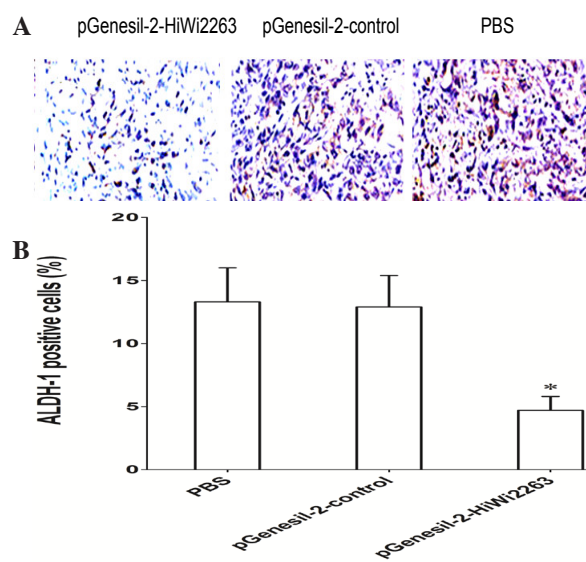
To determine whether the antitumor effect of Hiwi shRNA was associated with the inhibited expression of





**Figure 2. Immunohistochemical Analysis of Hiwi Expression in SSC<sup>lo</sup>Alde<sup>br</sup> Tumors after Treatment with shRNA Plasmids.** (A) Tumor tissues from nude mice inoculated with SSC<sup>lo</sup>Alde<sup>br</sup> cells were stained with the anti-Hiwi monoclonal antibody, Hiwi expression in tumor tissues was shown as positive reaction in the cytoplasm and the nuclei. Original magnification,  $\times 400$ . pGenesil-2-Hiwi2263, mice treated with the pGenesil-2 plasmids carrying Hiwi2263 shRNA; pGenesil-2-control, mice treated with the pGenesil-2 plasmids carrying negative control shRNA; PBS, mice treated with the vehicle PBS. (B) The immunostained slides were evaluated on light microscopy using a total magnification of 400X and a 10\*10 square grid placed in the ocular. The tumor cells were considered Hiwi positive when they displayed a distinct cytoplasmic and nuclear reaction. The percentage of Hiwi positive tumor cells was calculated as Hiwi labelling index (LI) = positive labeled cell/counting 500 tumor cells\*100%. Results were expressed as means  $\pm$  SEM of three experiments. \* $P < 0.05$  compared with the pGenesil-2-control group or the PBS group

Hiwi, tumors were harvested at the end of the treatment and analyzed by immunohistochemical staining with antibodies to Hiwi and ALDH-1. As shown in Figure 2A, the Hiwi protein was expressed in the cytoplasm and nuclei. Quantitation of Hiwi immunoreactivity showed that it was less significant in pGenesil-2-Hiwi2263 group compared with the pGenesil-2-control group or PBS group (Hiwi labeling index: 6.8% vs. 23.2% vs. 25.6%,  $P < 0.05$ , Figure 2B). Furthermore, a significant decrease in ALDH1 positive cells was observed in tumors from pGenesil-2-Hiwi2263 -treated animals, as seen by ALDH-1 staining. The percentage of ALDH-1-positive tumor cells was 13.3% in mice treated with the vehicle PBS and 12.9% in mice treated with pGenesil-2-control plasmids, by contrast, 4.7% of tumor cells in mice treated with the vehicle pGenesil-2-Hiwi2263 plasmids were ALDH-1 positive (Figure 3A and B). Considering that ALDH-1 is a confirmative marker of SSC<sup>lo</sup>Alde<sup>br</sup> cells, these findings indicated that treatment with the plasmids carrying with the Hiwi knockdown shRNA (pGenesil-2-Hiwi2263) will inhibit the Hiwi expression and attenuate the number of SSC<sup>lo</sup>Alde<sup>br</sup> cells in vivo, which then suppressed the tumor growth. Thus, the antitumor activity of Hiwi shRNA was related with the impaired expression of Hiwi in nude mice.



**Figure 3. ALDH-1 Expression in SSC<sup>lo</sup>Alde<sup>br</sup> Tumors after 14 Days of Plasmids Treatment.** (A) Tumor tissues from nude mice inoculated with SSC<sup>lo</sup>Alde<sup>br</sup> cells were stained with the anti-ALDH-1 monoclonal antibody, ALDH-1 expression in tumor tissues was shown as positive reaction in the cytoplasm and the nuclei. Original magnification,  $\times 400$ . (B) The immunostained slides were evaluated on light microscopy using a total magnification of 400X and a 10\*10 square grid placed in the ocular. The tumor cells were considered ALDH-1 positive when they displayed a distinct cytoplasmic and nuclear reaction. The percentage of ALDH-1 positive tumor cells was calculated as ALDH-1 labelling index (LI) = positive labeled cell/counting 500 tumor cells\*100%. Results were expressed as means  $\pm$  SEM of three experiments. \* $P < 0.05$  compared with the pGenesil-2-control group or the PBS group

## Discussion

Lung cancer stem cells had been isolated and considered to be responsible for drug resistance, tumor relapse and metastasis (Eramo et al., 2008). The stem cell-associated Hiwi gene, which is a human homologue of the Piwi family, plays an important role in the development of a variety of human cancers (Liu et al., 2006; Grochola et al., 2008). In the present study, we tested the possibility of using RNA interference as a therapeutic agent against human lung cancer.

RNA interference is an excellent strategy for gene silencing (Zamore et al., 2000). Studies showed that transfection of synthetic 21-nucleotide small interfering RNA (siRNA) duplexes into mammalian cells efficiently inhibits endogenous gene expression in a sequence-specific manner (Zamore et al., 2000). However, phenotypic changes induced by siRNAs persist for at most 1 week, which limits their utility. Short hairpin RNAs (shRNAs) driven by polymerase III promoters have been investigated as an alternative strategy to more stably suppress gene expression, and such constructs with well-defined initiation and termination sites have been used to produce various small RNA species that inhibit the expression of genes with diverse functions in mammalian cell lines (Yu et al., 1995; Chong et al., 2001; Paul et al., 2002). We have used RNA interference to investigate the role of the Hiwi gene in the proliferation and apoptosis

of lung cancer stem cells, and found that shRNA against Hiwi gene could significantly suppressed the proliferation and promoted the apoptosis of lung cancer stem cells. To test whether Hiwi knockdown could inhibit tumor growth in vivo, the nude mouse xenograft tumor models were established by subcutaneously inoculation of lung cancer stem cells. After the tumor size reached 8mm in diameter, the shRNA plasmids were administered via the tail vein. The results showed that the tumor growth was significantly attenuated by Hiwi knockdown. Further immunohistochemistry analyses confirmed that the Hiwi expression in xenograft tumor tissues from shRNA against Hiwi group was remarkably impaired. Moreover, delivery of Hiwi shRNA plasmids resulted in a significant suppression in ALDH-1 positive cells in xenograft tumor samples. Since ALDH-1 is a confirmative marker of the lung cancer stem cells (Liang & Shi, 2012), our data suggest that delivery of shRNA-mediated Hiwi gene silencing plasmids will inhibit the Hiwi expression in lung cancer stem cells, which resulted in the decreased number of lung cancer stem cells and suppressed tumor growth in nude mice. Thus, the current study clearly indicates that Hiwi does play a crucial role in the regulation of lung cancer stem cell growth and Hiwi shRNA plasmids possess effective antitumor activity against lung cancer.

Preclinical studies confirm that RNAi techniques can be used to silence cancer-related targets (Cioca et al., 2003; Scherr et al., 2003). In vivo studies have also shown favorable outcomes by RNAi targeting of components critical for tumor cell growth (Li et al., 2003), metastasis (Duxbury et al., 2004), angiogenesis (Filleur et al., 2003), and chemoresistance (Nakahira et al., 2007). However, the current knowledge about the inhibition of tumor cell proliferation by systemic treatment of tumor-bearing animals with shRNA plasmids is very limited. Our preliminary data confirmed the efficacy and offered some new sights for RNAi therapeutics with shRNA. Moreover, no significant adverse effects for the delivery method were observed in the present study, which proved its safety.

Cancer stem cells are considered to be responsible for the insensitivity or relapse of tumors to antineoplastic treatments such as chemotherapy and radiation (Clarke et al., 2006; Visvader et al., 2008). In the present study, Hiwi shRNA successfully knocked down expression of Hiwi protein in xenograft tumor samples and inhibited the proliferation and growth of lung cancer stem cells. Therefore, Hiwi RNAi vectors could be used in combination with other classical anticancer therapies (radio- and chemotherapy) to enhance the therapeutic effects.

In conclusion, we demonstrated for the first time that U6 promoter-driven shRNAs targeted against Hiwi gene can effectively suppress tumor growth in nude mice when administered intravenously. The results suggest that direct targeting of lung cancer stem cell Hiwi gene had major effects on their ability to grow in vivo, which provides evidence that directed delivery of Hiwi inhibition using RNAi technology would serve to suppress the growth of lung cancer stem cells in vivo and targeting Hiwi gene could be of use to treat lung cancer. However, the study was conducted in immune compromised mice, it will be

important to also study the effect of Hiwi knockdown in immunocompetent mice. Moreover, future studies should also focus on whether silencing of Hiwi expression using Hiwi shRNA is useful as a novel biotherapy strategy or an effective adjuvant therapy to chemotherapy for lung cancer patients.

## Acknowledgements

This work was supported by the Sanya Medical and Health of Science and Technology Innovation Project (No. YW1210 and YW1201), Hainan provincial health bureau's medical research (No. QW2012 PT-36), Heilongjiang Health Department (No. 2011-169) and the Project of Heilongjiang Science and Technology Department (No. QC2012C050). The author(s) declare that they have no competing interests.

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