

## RESEARCH ARTICLE

# Overexpression of Hiwi Promotes Growth of Human Breast Cancer Cells

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### Abstract

The Piwi subfamily comprises two argonaute (Ago) family proteins, which are defined by the presence of PAZ and Piwi domains, with well known roles in RNA silencing. Hiwi, a human Piwi subfamily member, has been shown to play essential roles in stem cell self-renewal and gametogenesis. Recently, accumulating reports have indicated that abnormal hiwi expression is associated with poorer prognosis of multiple types of human cancers, including examples in the breast. However, little is known about details of the oncogenic role of hiwi in breast cancers. In present study, we confirmed overexpression of hiwi in breast cancer specimens and breast cancer cell lines at both mRNA and protein levels. Thus both RT-qPCR and Western blot data revealed significantly higher hiwi in intratumor than peritumor specimens, overexpression being associated with tumor size, lymph node metastasis and histological grade. Hiwi overexpression was also identified in breast cancer cell lines, MDA-MB-231 and MCF-7, and gain-of-function and loss-of-function strategies were adopted to identify the role of hiwi in the MCF-7 cell growth. Results demonstrated that hiwi expression in MCF-7 cells was significantly up- or down- regulated by the two strategies. We next evaluated the influence of hiwi overexpression or knockdown on the growth of breast cancer cells. Both cell count and colony formation assays confirmed promoting roles of hiwi in MCF-7 cells, which could be inhibited by hiwi specific blockage by siRNAs. In summary, the present study confirmed overexpression of hiwi in breast cancer specimens and breast cancer cell lines, and provided evidence of promotion by hiwi of cell growth. The results imply an oncogenic role of hiwi in breast cancers.

**Keywords:** Hiwi - growth - human breast cancer cells

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### Introduction

Breast cancer leads the cause of death among solid tumours in women, with an increasing incidence, especially among younger woman (van Diest et al., 2004). Presently, the disease will affect 12% of all women and about 30-40% of patients will die from metastatic disease despite radical surgery (Brown et al., 2011). And several risk factors have been recognized, such as early menarche, late menopause, nulliparity, and positive family history (Purrington et al., 2014). However, there are at present no realistic options for primary prevention in patients, except hereditary breast cancer related genes, such as BRCA1 and BRCA2 (Marcus et al., 1996), PTEN (Cowden's disease) (Eng et al., 1994), and p53 (Li Fraumeni syndrome) (Magnusson et al., 2012). Recently, multiple molecules have been recognized as pro-oncogenic factors for breast cancers by the universal screen methods basing genomics, transcriptomics, proteomics (Ocana et al., 2008), or other methods. CYP3A4 expression has been recognized as a risk factor of breast cancer in Mexican women (Floriano-

Sanchez et al., 2014). ESR1 and PGR have been reported to be associated with ductal and lobular breast cancer (Medina-Jaime et al., 2014) and Expression of EMSY, a Novel BRCA2-link Protein, has been confirmed to be associated with lymph node metastasis of breast cancer (Madjd et al., 2014). Most of these factors were utterly incompetent as diagnostic or therapeutic targets, because they are basing on traditional models of tumorigenesis which suggest that malignancies develop in a series of stepwise genetic mutations culminating in the generation of cancer cells able to disseminate and metastasize (Klein et al., 2009). However, recent evidence strongly suggests that metastasis may be an early event in tumorigenesis (Husemann et al., 2008, Weng et al., 2012). The cancer stem cell (CSC) hypothesis offers new insights into tumorigenesis and metastatic progression that may lead to more effective therapies to breast cancers.

There were two models for the development of CSCs, the cancer stem cell model and the clonal evolution model. In former model, random mutations that are accumulated by normal stem cells or their early progeny (Reya et

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al., 2001). By contrast, the clonal evolution model is largely based on the hypothesis that any undifferentiated or differentiated cell can accumulate mutations that lead to tumor formation and to the generation of clonal populations of cells within the tumor (Hochedlinger et al., 2006). Whatever the mechanism that leads to the production of cancer stem cells, an understanding of their biology is crucial given the high mortality rate that is associated with the presence of undifferentiated cancer cells within tumors. Breast tumors that are enriched in undifferentiated cancer cells are typically more aggressive and prone to metastasis, resistance to therapy and relapse events, which can occur either at the primary tumor site or at distant metastatic sites following an apparent remission or dormancy for several years (Brackstone et al., 2007). Thus, the therapeutic targeting of cancer stem cells promises to be clinically fruitful.

The hiwi gene is the human homolog of the piwi family, locating in 12q24.33 and encodes hiwi protein of 98.5 kDa (Qiao et al., 2002). The Piwi family represents the first class of genes known to be required for stem cell self-renewal in diverse organisms such as jellyfish, *Caenorhabditis elegans*, *D. melanogaster*, zebrafish, mouse and humans (Liu et al., 2006). Hiwi mRNA is present in human CD34 (+) haematopoietic progenitor cells, but not in more differentiated cell populations (Sharma et al., 2001). In addition to its function in stem cell renewal, overexpression of Hiwi in male germline cells correlates with the occurrence of seminomas, a testicular germ cell tumours (Qiao et al., 2002). Recently, accumulating reports have revealed that abnormal hiwi expression is associated with poorer prognosis of human malignant tumors, such as human esophageal squamous cell carcinoma (He et al., 2009), human adenocarcinoma of the pancreas (Grochola et al., 2008), human gastric cancer (Liu et al., 2006), and colorectal cancer (Oh et al., 2012). The alterations in mRNA expression of hiwi can increase the risk of tumour-related death in male human adenocarcinoma of the pancreas patients (Grochola et al., 2008); The expression of hiwi in the cytoplasm of esophageal cancer cells is significantly associated with higher histological grade, clinical stage and poorer clinical outcome (He et al., 2009); And the high degree of hiwi expression was significantly correlated with the lower degree of differentiation, deep invasion and perineural invasion (Oh et al., 2012). Moreover, the overexpression pattern of hiwi in gastric cancer tissues was similar to that of Ki67, which was used as a marker of growth of cancer cells. and the suppression of hiwi inhibited the growth of gastric cancer cells and induced cell cycle arrest in G2/M phase (Liu et al., 2006). Whereas, hiwi knockdown inhibits the growth of lung cancer in nude mice (Liang et al., 2013). These results suggest that hiwi may be involved in the tumorigenesis of various cancers and is a potential target for cancer therapy.

In present study, we evaluated the overexpression of hiwi in human breast cancer specimens, and in breast cancer cell lines, MDA-MB-231, MCF-7 and MCF-12A cells. Then, MCF-7 cells overexpressing hiwi, with a eGFP marker coexpressed was constructed, on the other hand, a lentivirus harboring shRNA targeting hiwi was

also rescued to knockdown hiwi. Then we investigated the influence of hiwi on the MCF-7 cell growth. It is implied that hiwi plays an important role in breast cancer cell growth and can be a potential target for anticancer therapy.

## Materials and Methods

### *Breast cancer specimens, Cell lines and culture*

47 human breast cancer specimens and 47 peritumor specimens (as control; at a distance  $\geq 10$ mm from the tumor edge) were resected from breast cancer patients, with complete clinico-pathologic data, who were registered in the China-Japan Union Hospital, Jilin University from April 2007 to Jun 2012, with informed consent and agreement. Clinico-pathological data of the patients were recorded prospectively, including the age at diagnosis, menstruation status, tumor size, axillary lymph node metastasis and histological grade. All fresh specimens were resected before being exposed to radiotherapy and chemotherapy, were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  post resection. And 10 peritumor specimens of breast cancers were used as control for hiwi expression in breast cancer cell lines. Present study has been approved by the medical ethics committee of China-Japan Union Hospital, Jilin University. Breast cancer cell lines, MDA-MB-231, MCF-7 and MCF-12A were purchased from Shanghai Bioleaf biotech co.Ltd (Shanghai, China) and were cultured at  $37^{\circ}\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$  in Dulbec-co's modified Eagle's medium (DMEM) or RP1640 medium (for MCF-12A) (both from Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; GIBCO, Rockville, MD, USA).

### *RNA extraction and quantitative real-time polymerase chain reaction*

Total RNA from the intra-tumor, peritumor specimens, or the breast cancer cell lines was extracted using the RNeasy plus mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions, and added with Rnase inhibitor (TaKaRa Bio Inc., Tokyo, Japan). Quantitative real-time RT-PCR (RT-qPCR) was performed using a SYBR PrimeScript RT-qPCR Kit (TaKaRa Bio Inc., Tokyo, Japan) according to the kit manual, with  $\beta$ -actin as an internal control. The primers for hiwi and  $\beta$ -actin were synthesized by Shanghai Sangon company (Shanghai, China) according to the reported sequences (Zhao et al., 2012). The RT-qPCR was performed with a Lightcycler 480 II (Roche, Mannheim, Germany). All data were normalized to  $\beta$ -actin and expressed as the fold change over control and calculated with the  $\Delta\Delta\text{Ct}$  method (Livak et al., 2001).

### *Western Blot Analysis for hiwi expression*

Breast cancer specimens for western blot analysis were homogenized before protein isolation. The homogenized breast specimens or cultured breast cancer cells were collected and lysed with a lysis reagent (Sigma-Aldrich, St. Louis, MO, USA), according to the manual, and were supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). Protein samples were

separated by 10% SDS-PAGE gel, and were transferred to a PVDF membrane. The hiwi and  $\beta$ -actin were detected, via successively using anti-hiwi or anti- $\beta$ -actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), a peroxidase-conjugated secondary antibody (Sigma, St. Louis, MO, USA) and the electrochemoluminescence (ECL) detection system (Amersham, Uppsala, Sweden) following the manufacturer's instructions.

#### Manipulation of hiwi level in MCF-7 cells

To generate a hiwi overexpressed MCF-7 cell line, the wild hiwi coding sequence and was amplified and cloned into the pcDNA3.1 (+) vector (Invitrogen, Carlsbad, CA, USA). And the Hiwi-2A-eGFP-pcDNA3.1 (+) or control pcDNA3.1(+) vectors was transfected into MCF-7 cells by lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The hiwi and eGFP positive cell clones were selected in the presence of 1.5mg/ml G418 (Sigma-Aldrich, St. Louis, MO, USA), and maintained in medium containing G418 at 1 mg/mL. The 50 nM hiwi specific siRNAs, siRNA-Hiwi 1, siRNA-Hiwi 2 or siRNA-Con (Sangon, Shanghai, China) was transfected with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) into the MCF-7 Hiwi (+) cells to abrogate the Hiwi expression.

#### Cell count assay and Colony formation assay

Cell count assay was performed as previously described (Li et al., 2013). Briefly, cells plated in 12-well plates and were incubated at 37°C for various periods of time and then trypsinized. The number of viable cells was counted in a hemocytometer with the use of trypan blue staining. For the colony formation assay, 500 cells in each group were seeded into a 12-well plate. After 6 days, cells were stained by 0.5% crystal violet (Sigma-Aldrich, St. Louis, MO, USA). And colonies were counted directly on the plate.

#### Statistical analysis

Statistical analyses were performed using SPSS16.0 software (IBM SPSS, Armonk, NY, USA). All data were expressed as mean $\pm$ standard error of the mean (SEM). The expression of hiwi in mRNA or protein level, the cell number or the colony number two groups were analyzed by Student's t test. A  $p < 0.05$  or less was considered statistically significant.

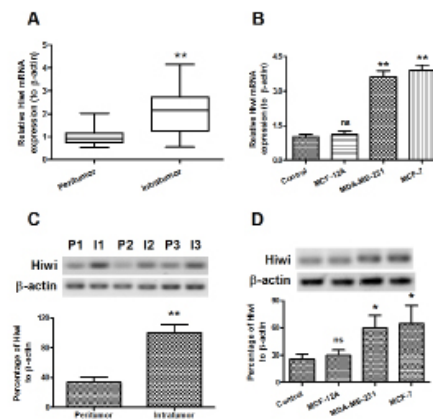
## Results

#### Overexpression of hiwi in breast cancer specimens and breast cancer cell lines, and is associated with the cancer malignance.

To identify a possible oncogenic role of hiwi in breast cancers, we determined the hiwi expression in breast cancer specimens and cell lines. Firstly, the RT-qPCR revealed a significant high hiwi level in the intratumor specimens than in the peritumor specimens (Figure 1A,  $2.092 \pm 0.237$  vs  $1.000 \pm 0.162$ ,  $p < 0.01$ , paired-samples  $t$ -test) from 47 breast cancer patients with complete clinico-pathological data. Then the correlation of hiwi overexpression with clinico-pathological variants were analyzed. It was shown in Table 1, that the hiwi expression

was not associated with the age and menopause ( $p = 0.2536$  and  $0.3042$  respectively). However, there was a significant association of hiwi overexpression with the tumor size, lymph node metastasis and histological grade. The hiwi expression in patients with larger size, axillary lymph node metastasis or higher histological grade was significantly higher ( $p = 0.0360$ ,  $0.0143$  or  $0.0093$ , respectively). Secondly, we re-evaluated the hiwi expression in protein level by western blot analysis in 15 intratumor specimens and 15 peritumor specimens. As shown in Figure 1C, the protein level of hiwi in the intratumor specimens was also significantly higher than in the peritumor specimens ( $p < 0.01$ , paired-samples  $t$ -test).

And the significant high hiwi mRNA level was also



**Figure 1. Overexpression of Hiwi Protein in Breast Cancer Specimens and Cell Lines.** A) Relative mRNA level of Hiwi in the intratumor (n=47) or peritumor tissues (n=47) of breast cancers, revealing by quantitative real-time RT-PCR; B) Relative mRNA level of Hiwi in the breast cancer cell lines; C) Overexpression of Hiwi in protein level in the intratumor (n=15) or peritumor tissues (n=15) of breast cancers, revealing by western blot analysis. D) Overexpression of Hiwi in protein level in breast cancer cell lines, revealing by western blot analysis. Statistical significance was considered with a  $p < 0.05$  or less, \*  $p < 0.05$ , \*\*  $p < 0.01$ , ns, no significance

**Table 1. Association of Hiwi Expression with Clinicopathological Variants in Breast Cancers**

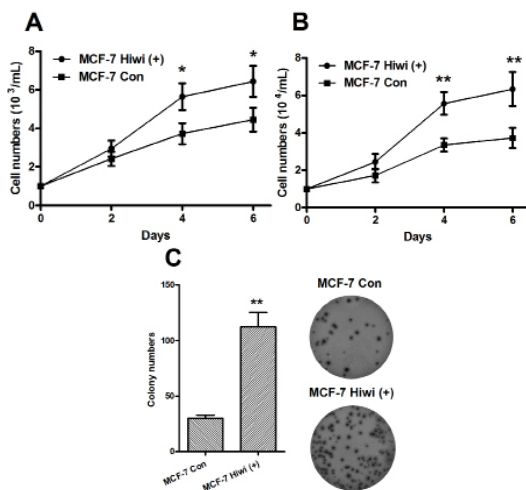
Clinicopathological variants	n	Relative Hiwi level*	p-value
Age (yr)			
≤50	22	1.986 $\pm$ 0.244	0.2536
>50	25	2.185 $\pm$ 0.225	
Menopause			
Premenopausal	23	2.165 $\pm$ 0.241	0.3042
Postmenopausal	24	2.022 $\pm$ 0.228	
Tumor size (cm)			
T1	0	/	0.0360
T2	13	1.825 $\pm$ 0.203	
T3	19	2.131 $\pm$ 0.219	
T4	15	2.275 $\pm$ 0.315	
Axillary lymph node metastasis			
Negative	21	1.881 $\pm$ 0.352	0.0143
Positive	26	2.263 $\pm$ 0.197	
Histological grade			
I/II	19	1.732 $\pm$ 0.294	0.0093
III	28	2.336 $\pm$ 0.210	

\*: The Relative Hiwi level was normalized to  $\beta$ -actin, and relative to the hiwi expression to each peritumor tissue (which was designated as "1").

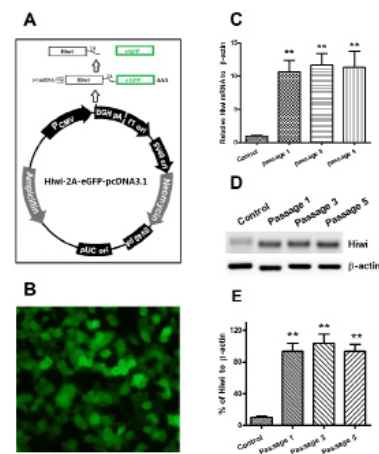
found in breast cancer cell lines, MDA-MB-231, MCF-7, but not in MCF-12A, compared to the primary breast cells (Figure 1B,  $p < 0.01$  for MDA-MB-231 or MCF-7,  $p > 0.05$  for MCF-12A, paired-samples  $t$ -test). And Figure 1D also indicated that there was a significant high level of hiwi expression in protein level in both MDA-MB-231 and MCF-7 cells ( $p < 0.05$ , unpaired-samples  $t$ -test), but not in MCF-12A cells ( $p > 0.05$ , unpaired-samples  $t$ -test). Taken together, we confirmed the overexpression of hiwi in breast cancer specimens and breast cancer cell lines.

**Construction of MCF-7 stable cell line overexpressing hiwi**

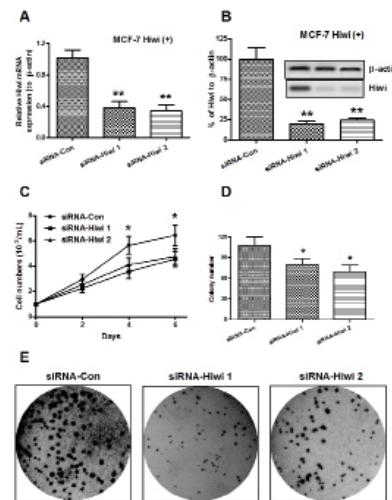
To identify the oncogenic role of hiwi in breast cancer cells, we generate an hiwi and eGFP co-expressing MCF-7 cell line. Hiwi and eGFP cDNA were amplified and then over-lapped with a 2A peptide coding sequence between them, and then were cloned into the eukaryotic expression vector, pcDNA3.1 (+). As shown in Figure 2A, the co-expressing method with 2A peptide guarantees both molecules to be transcribed into one mRNA molecule, but be translated into two separate proteins (Szymczak et al., 2004, Szymczak-Workman et al., 2012). MCF-7 cells were transfected with the recombinant hiwi-2A-Egfp-pcDNA3.1 (+) plasmid and selected under the G418 pressure. Post serial passages with 1.5 mg/mL G418, MCF-7 Hiwi (+) cells were isolated and propagated, with a eGFP positive (Figure 2B). Then the overexpression of hiwi were determined. A stabilized Hiwi overexpression in mRNA level in MCF-7 Hiwi (+) cells were determined by the RT-qPCR method with the hiwi specific primers. A significantly higher level of hiwi mRNA was observed in the MCF-7 Hiwi (+) cells with various passages ( $t$ -test,  $p < 0.01$  respectively, compared to the control cells, MCF-7 cells) (Figure 2C). And the hiwi overexpression in protein



**Figure 3. Overexpression of Hiwi Promotes the Growth of Human Breast Cancer Cells, MCF-7.** **A)** Significantly high growth of MCF-7 Hiwi (+) cells than the MCF-7 cells after an inoculation of 103 /mL cells. **B)** Significantly high growth of MCF-7 Hiwi (+) cells than the MCF-7 cells after an inoculation of 104 /mL cells. **C)** Difference in cell growth by colony formation assay between the MCF-7 Hiwi (+) cells and MCF-7 cells. The experiments were performed separately in triplicate. Statistical significance was shown as \*  $p < 0.05$ , \*\*  $p < 0.01$



**Figure 2. Construction of MCF-7 Stable Cell Line Overexpressing Hiwi.** **A)** Schematic diagram of Hiwi and eGFP co-expression with a 2A peptide sequence, all of which were cloned into pcDNA3.1 vector; **B)** The GFP-positive MCF-7 cells overexpressed Hiwi. **C)** RT-qPCR analysis of the over-expressed Hiwi mRNA in MCF-7 Hiwi (+) cell line post serial passages, compared to  $\beta$ -actin. **D and E)** Western blot indicated Hiwi overexpression in protein level in serially propagated MCF-7 Hiwi (+) stable cell line, normalized to  $\beta$ -actin. Statistical significance was showed as \*\*  $p < 0.01$ . Data represent the mean  $\pm$  SEM of three independent experiments



**Figure 4. RNA Interference Blocks Hiwi Expression and Inhibits the Growth of MCF-7 Hiwi (+) cells.** **A)** Significant suppression by siRNA of the hiwi mRNA in MCF-7 Hiwi (+) cells 24 h post the transfection of hiwi specific siRNAs, compared to the siRNA control, revealing by quantitative real-time RT-PCR. **B)** Hiwi expression in protein level in MCF-7 Hiwi (+) cells 48 h post transfection of hiwi specific siRNAs or siRNA control, revealing by western blot analysis.  $\beta$ -actin was used as an internal control. **C)** Significant less growth of MCF-7 Hiwi (+) cells in siRNA-Hiwi 1 or shRNA-Hiwi 2 group than in control siRNA group. The growth curve of both cells were evaluated by cell count assay. **D and E)** Cell growth by colony formation assay in MCF-7 Hiwi (+) cells with or without Hiwi knockdown by siRNAs. The experiments were performed separately in triplicate. Statistical significance was shown as \*  $p < 0.05$ , \*\*  $p < 0.01$



level was stabilized in the MCF-7 Hiwi (+) cells (t test,  $p < 0.01$ , respectively) (Figure 2 D and E).

#### *Hiwi overexpression promotes the growth of breast cancer cells, MCF-7*

We then evaluated the influence of hiwi overexpression on the growth of breast cancer cells. The growth of breast cancer cell *in vitro* was assessed by cell count assay and colony formation assay. Firstly, the cell count assay was performed for cell counting every other day for three times post cell inoculation. As shown in Figure 3A, when inoculated with  $10^3$ /mL, MCF-7 Hiwi (+) cells grew more efficiently than MCF-7 cells, particularly at 4 or 6 day post inoculation, with a significant difference ( $5.64 \pm 0.70 \times 10^3$  /mL for MCF-7 Hiwi (+) cells and  $3.72 \pm 0.54 \times 10^3$  /mL for MCF-7 cells at 4 day post inoculation, and  $6.43 \pm 0.81 \times 10^3$  /mL for MCF-7 Hiwi (+) cells and  $4.45 \pm 0.62 \times 10^3$  /mL for MCF-7 cells.  $p < 0.054$ ,  $p < 0.05$ , respectively). The growth difference was reconfirmed with an inoculated with  $10^4$ /mL, the growth of MCF-7 Hiwi (+) cells was also significantly more efficient than MCF-7 cells (Figure 3B;  $p < 0.01$ , respectively). Furthermore, the colony formation for both MCF-7 Hiwi (+) and MCF-7 cells was evaluated. As shown in Figure 3C, there was more colonies formed by MCF-7 Hiwi (+) cells than MCF-7 cells, post an inoculation of same cell number ( $p < 0.01$ ). Thus, we confirmed the promotory role of hiwi to the growth of breast cancer cells by both kinds of assays.

#### *RNAi-mediated hiwi knockdown blocks the promotion of overexpressed hiwi to the MCF-7 cell growth*

To further confirm the promotion by hiwi to the growth of breast cancer cells, we knockdown the hiwi with hiwi specific siRNAs in the MCF-7 Hiwi (+) cells, and then to determine the influence of hiwi knockdown on the breast cancer cell growth. Then MCF-7 Hiwi (+) cells were transfected with the 30 nM siRNA-Hiwi 1, siRNA-Hiwi 2, or siRNA-Con according to the product manual. Figure 4A and 4B indicated that both siRNAs significantly blocked the hiwi expression in both mRNA and protein levels, compared to the siRNA-Con ( $p < 0.01$  respectively, unpaired t test). We then evaluated the influence of hiwi knockdown on the growth of breast cancer cells. by cell count assay and colony formation assay. Firstly, the cell count assay was performed for cell counting every other day for three times post the inoculation of  $10^3$ /mL MCF-7 Hiwi (+) cells. As shown in Figure 4C, both siRNAs significantly inhibited the MCF-7 Hiwi (+) cell growth at 4 or 6 day post inoculation, compared to the siRNA-Con (either  $p < 0.05$ , unpaired t test). As shown in Figure 4D and 4E, the numbers of MCF-7 Hiwi (+) formed colonies in both siRNA-Hiwi 1 and siRNA-Hiwi 2 groups were significantly less than in the control siRNA group (either  $p < 0.05$ , unpaired t test). Thus, we reconfirmed the promotory role of hiwi to the growth of breast cancer cells by the loss-of-function strategy.

## Discussion

As a member of Argonaute (Ago) family, the hiwi protein also contains a conserved architecture with a

PAZ motif and Piwi motif (Cerutti et al., 2000, Yan et al., 2003). And the Piwi motif has a structural homology to RNase H endonuclease (Parker et al., 2004). Proteins with Piwi domains have been described as components of ribonucleoprotein complexes that act in the microRNA/RNA interference pathway of gene silencing (Fetzer et al., 2002, Lingel et al., 2005). Recently, it was shown that the Piwi gene family are associated with a new class of small RNAs so-called 'piwi-interacting RNAs' (piRNAs) in mammalian spermatogenesis (Hall et al., 2005, Houwing et al., 2007, Hutvagner et al., 2008, Peters et al., 2007), which is one of gene expressing regulatory small non-coding RNAs (ncRNAs). Small ncRNAs are naturally conserved regulators of gene expression described in almost all eukaryotic species including humans (Elbashir et al., 2001, Meister et al., 2004, Sana et al., 2012). To perform their effector functions, small ncRNAs must be incorporated into Ago complexes and form highly specialized small-RNA-binding modules in RNA-silencing pathways. However, it is not clear how the ribonucleoprotein-like molecule promotes the tumorigenesis.

More recently, professor Yang and his colleagues have confirmed that the hiwi expression inhibition could significantly reduce the tumor growth in a xenograft mouse model. Further immunohistochemistry analyses confirmed that the hiwi blockage also induced a significant suppression in ALDH-1 positive cells in xenograft tumor samples (Liang et al., 2013). Since ALDH-1 is a confirmative marker of the lung cancer stem cells (Liang et al., 2012), it implied that delivery of shRNA-mediated Hiwi gene silencing resulted in the decreased number of lung cancer stem cells and suppressed tumor growth in nude mice. Thus, hiwi may play a crucial role in the regulation of lung cancer stem cell growth.

In present study, we confirmed the overexpression of hiwi in breast cancer specimens and breast cancer cell lines in both mRNA and protein levels. Both RT-qPCR and western blot analysis revealed a significant high hiwi level in intratumor specimens than in the peritumor specimens. And the hiwi overexpression was significantly associated with the tumor size, lymph node metastasis and histological grade. Moreover, the hiwi overexpression was also identified in breast cancer cell lines, MDA-MB-231, MCF-7, but not in MCF-12A. Then, both the gain-of-function and loss-of-function strategies were adopted to identify the role of hiwi in the MCF-7 cell growth. Results demonstrated that the hiwi expression in MCF-7 cells was significantly up- or down- regulated by the two strategies. We next evaluated the influence of hiwi overexpression or knockdown on the growth of breast cancer cells. Both cell count assay and the colony formation assay confirmed the promotory role of hiwi in MCF-7 cells, and the promotion could be inhibited by the hiwi specific blockage by siRNAs. Taken together, our study implied an oncogenic role of hiwi in breast cancer. However, the mechanism of the oncogenic regulation by hiwi needs to be further recognized.

In summary, present study confirmed the overexpression of hiwi in breast cancer specimens and breast cancer cell lines, identified the promotion of hiwi to the growth

of breast cancer cells, using both the gain-of-function and loss-of-function approaches: Overexpressed hiwi promoted MCF-7 cell growth, while hiwi knockdown by RNAi method inhibited the promotion by hiwi. It implied the oncogenic role of hiwi in breast cancers.

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