January 2014

The Oncogenic Role Of Piwi Proteins In Breast Carcinogenesis

Donghai Liang
Yale University, donghai.liang89@gmail.com

The Oncogenic Role of PIWI Proteins in Breast Carcinogenesis

By

Donghai Liang

Bachelor of Science 2012

Peking University, College of Life Science

A Thesis Presented to

The Faculty of the Department of Environmental Health Sciences

Yale University

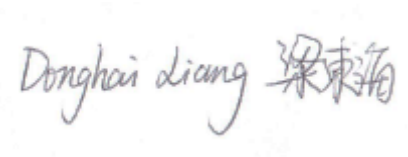
In Candidacy for the Degree of

Master of Public Health

Committee Members

Yong Zhu, Ph.D. & Yawei Zhang, Ph.D.

Permission for photocopying, microfilming, or computer electronic scanning of "The Oncogenic Role of PIWI Proteins in Breast Carcinogenesis" for the purpose of individual scholarly consultation or reference is hereby granted by the author. This permission is not to be interpreted as affecting publication of this work or otherwise placing it in the public domain, and the author reserves all rights of ownership guaranteed under common law protection of unpublished manuscripts.



_____

Signature of Author

05/01/2014

_____

Date

ABSTRACT

As the leading source of cancer death among women worldwide, breast cancer has experienced a steady increase in prevalence in recently years, a trend that is most evident in industrialized nations. One possible explanation for this epidemic phenomenon is the exposure to light at night through shiftwork, which has been linked to both aberrant DNA methylation and increased risk of breast cancer development. Based on a whole-genome methylation analysis on a selected sample of the Danish Diet, Cancer and Health prospective cohort, we observed statistically significant hypomethylation in the PIWIL1 gene, a member of the PIWI protein family, among night shiftworkers. Upon analysis of expression microarray data from the Oncomine database, we observed a trend of PIWI gene overexpression in breast cancer patients relative to healthy controls. This finding was confirmed when we measured the endogenous expression of PIWI genes in breast cell lines, which revealed overexpression of PIWIL1 and PIWIL4 in breast cancer cells relative to normal breast cells. To further elucidate the functional link between PIWI gene expression and breast cancer, we knocked down PIWIL1 and L4 in breast cancer cells and observed a substantial decrease in the rate of cellular proliferation, which suggests that these genes may play a role in the breast carcinogenic process. Future in-depth investigation into the function of PIWI genes is warranted to gain a more comprehensive understand of their role in breast carcinogenesis.

Acknowledgements

This project receives the great grant support from the National Institute of Environmental Health Sciences (NIEHS, Grant Number ES018915), Yale University and the Danish Cancer Society. I would like to acknowledge Dr. Yong Zhu of the Yale School of Public Health for his expert advice and insightful direction in the conception and writing of this manuscript. I want to also thank Dr. Alan Fu, Daniel Jacobs and Dr. Qin Qin for their technical support and the revision of this manuscript. This project also received the generous support from the Cynthia Barnett Cancer Prevention Fund.

Table of Contents

LIST OF FIGURES

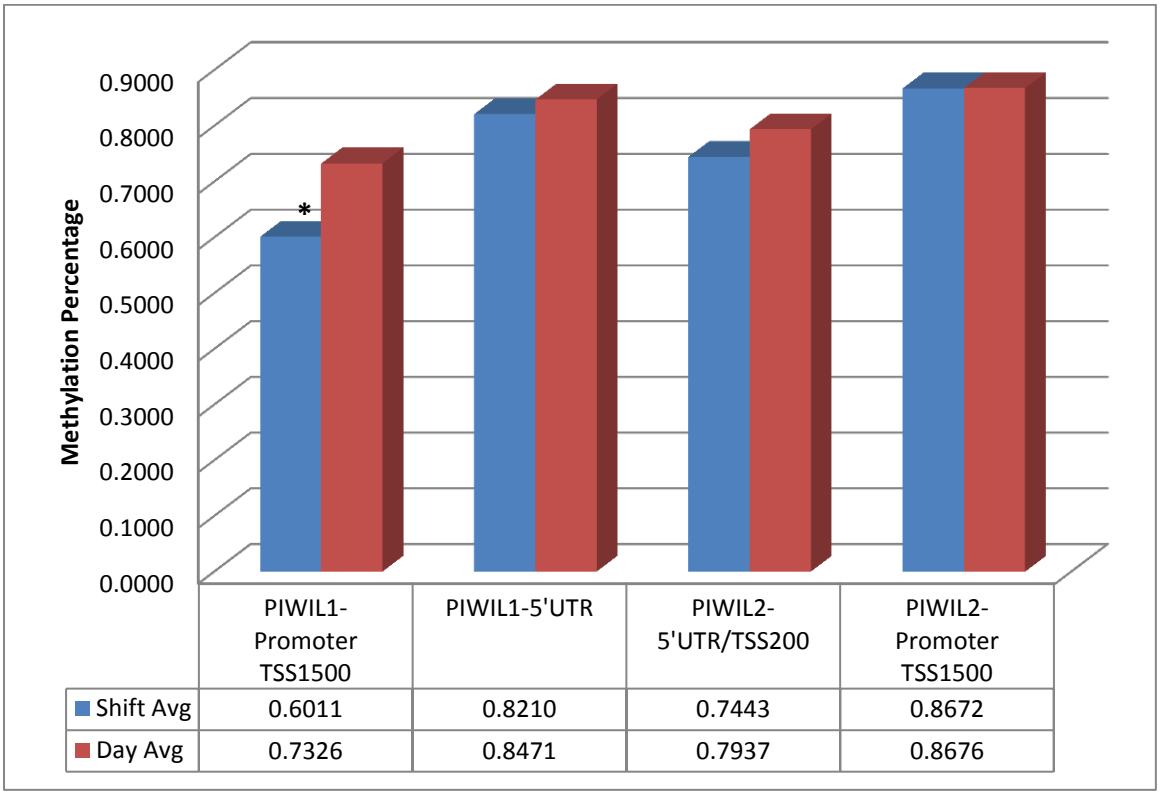


Figure 1 Methylation indices for day workers and shiftworkers at PIWI-associated CpG sites. We observed a 13.14% decrease in methylation at a CpG site within PIWIL1's proximal promoter region ($Q=2.32*10^{-6}$), a 2.61% decrease at a CpG site within PIWIL1's 5'UTR region ($Q>0.05$), a 4.94% decrease at a CpG site within PIWIL2's 5'UTR region ($Q>0.05$), and a 0.04% decrease at a CpG site within PIWIL2's proximate promoter region ($Q>0.05$).

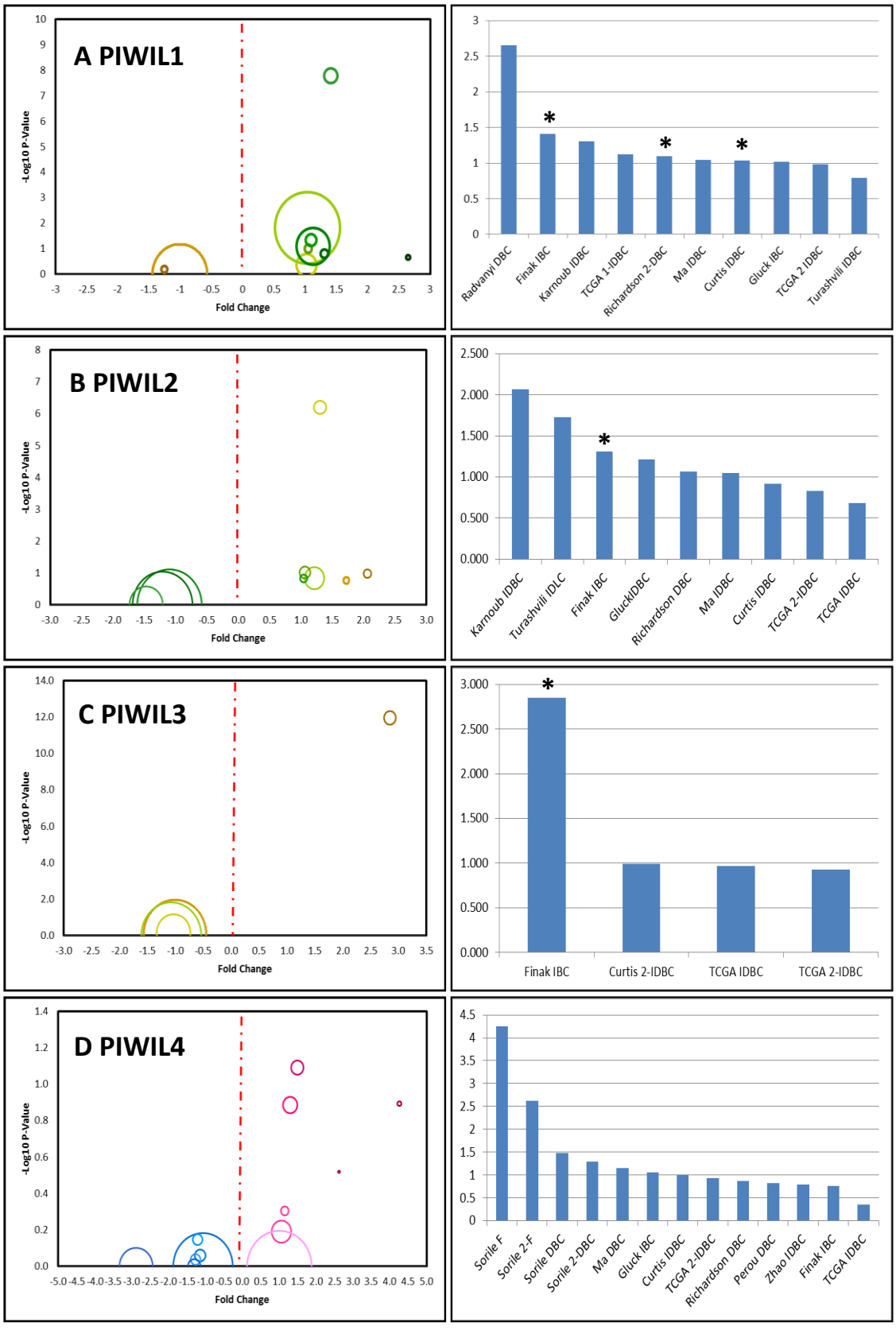

A PIWIL1
-Log10 P-Value
Fold Change
Radvanyi DBC
Finak IBC
Karnoub IDBC
TCGA 1-IDBC
Richardson 2-DBC
Ma IDBC
Curtis IDBC
Gluck IBC
TCGA 2 IDBC
Turashvili IDBC
B PIWIL2
-Log10 P-Value
Fold Change
Karnoub IDBC
Turashvili IDLC
Finak IBC
GluckIDBC
Richardson DBC
Ma IDBC
Curtis IDBC
TCGA 2-IDBC
TCGA IDBC
C PIWIL3
-Log10 P-Value
Fold Change
Finak IBC
Curtis 2-IDBC
TCGA IDBC
TCGA 2-IDBC
D PIWIL4
-Log10 P-Value
Fold Change
Sorile F
Sorile 2-F
Sorile DBC
Sorile 2-DBC
Ma DBC
Gluck IBC
Curtis IDBC
TCGA 2-IDBC
Richardson DBC
Perou DBC
Zhao IDBC
Finak IBC
TCGA IDBC

Figure 2 PIWI gene expression in breast tumor tissues relative to normal tissues. We queried the Oncomine database for breast cancer studies with PIWI gene expression data. 22 out of the 36 analyses found showed higher expression in one or more PIWI genes. Analyses exhibiting expression differences at P-values < 0.05 are marked with '*'. The size of each circle is scaled by the sample size of the corresponding analysis. Four subtypes of breast cancer are included in the analyses: invasive ductal breast carcinoma (IDBC), invasive breast carcinoma (IBC), ductal breast carcinoma (DBC), invasive lobular breast carcinoma (ILBC) and fibroadenoma (F). Figures 2A, 2B, 2C and 2D refer to the expression analyses for PIWIL1, PIWIL2, PIWIL3 and PIWIL4, respectively, in breast tumors compared to normal breast tissue.

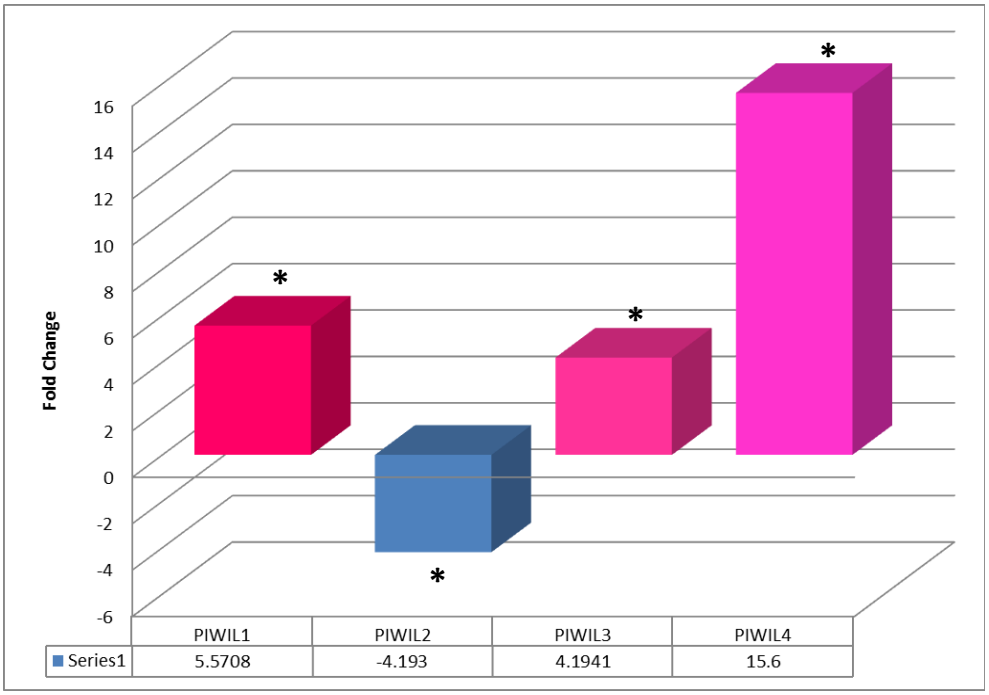


Figure 3 PIWI Gene expression differences in MCF7 breast cancer cells compared to MCF10a normal cells. PIWI mRNAs expression levels in MCF7 cells and MCF10a cells were measured by quantitative real-time PCR performed in technical triplicate. RNA quantity was normalized using the HPRT housekeeping gene and quantified using the $2^{-\Delta\Delta Ct}$ method. Of the four PIWI Genes, PIWIL1, PIWIL3 and PIWIL4 were 5.57 times, 4.194 times and 15.6 times overexpressed in MCF7 cells relative to that in MCF10a cells, while PIWIL2 was 4.193 times underexpressed in breast cancer cells compared to control. All of the expression differences achieved statistical significance ($P<0.01$).

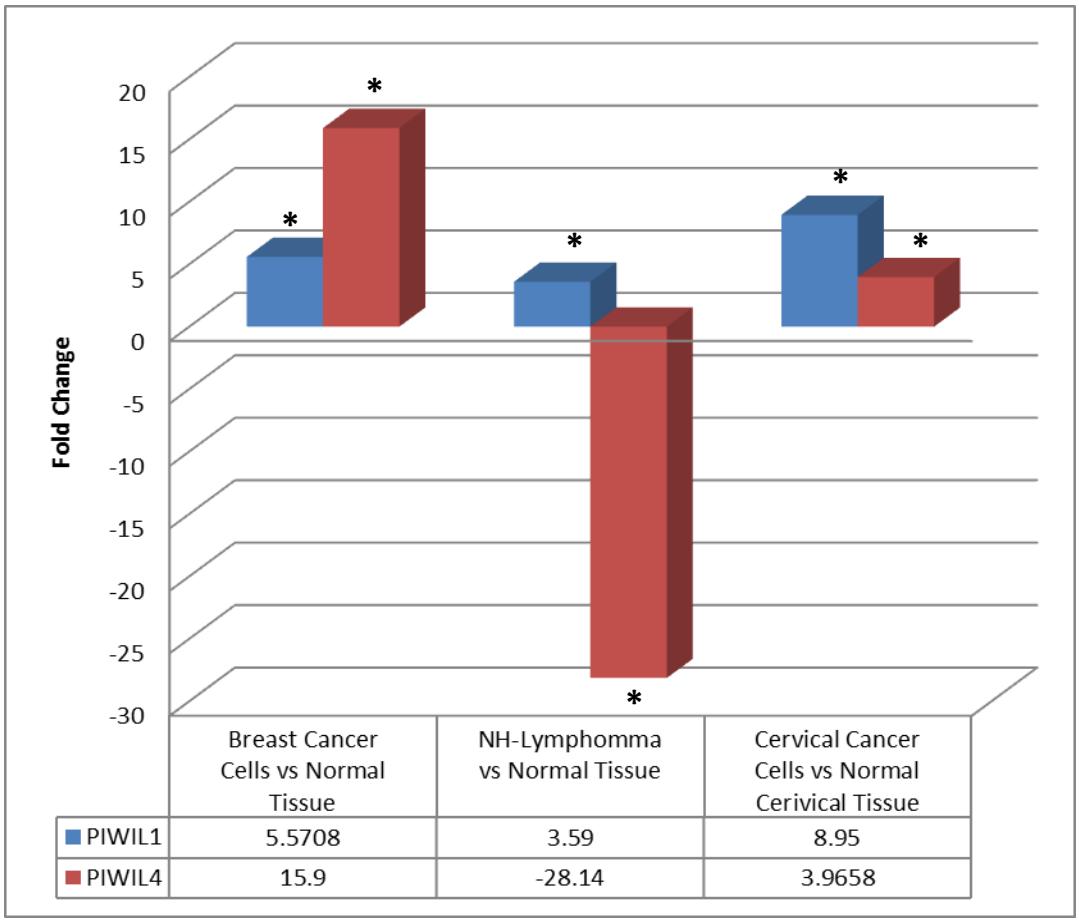


Figure 4 PIWIL1/PIWIL4 expression differences in various cancer cell lines compared to normal cell lines. The mRNA expression of PIWIL1 and PIWIL4 were measured by quantitative real-time PCR performed in technical triplicate in three cancer types: breast cancer (MCF7 vs. MCF10a), cervical cancer (Hela vs. END1) and non-Hodgkin's lymphoma (Farrage vs. RPM1788). In cervical cancer cell lines, both PIWIL1 and PIWIL4 were overexpressed compared to normal cells (8.95- and 3.97-fold, respectively). In non-Hodgkin's lymphoma, PIWIL1 was 3.59 times overexpressed compared to the normal cells while PIWIL4 was 28.14 times underexpressed. All of the expression differences achieved statistical significance ($P<0.01$).

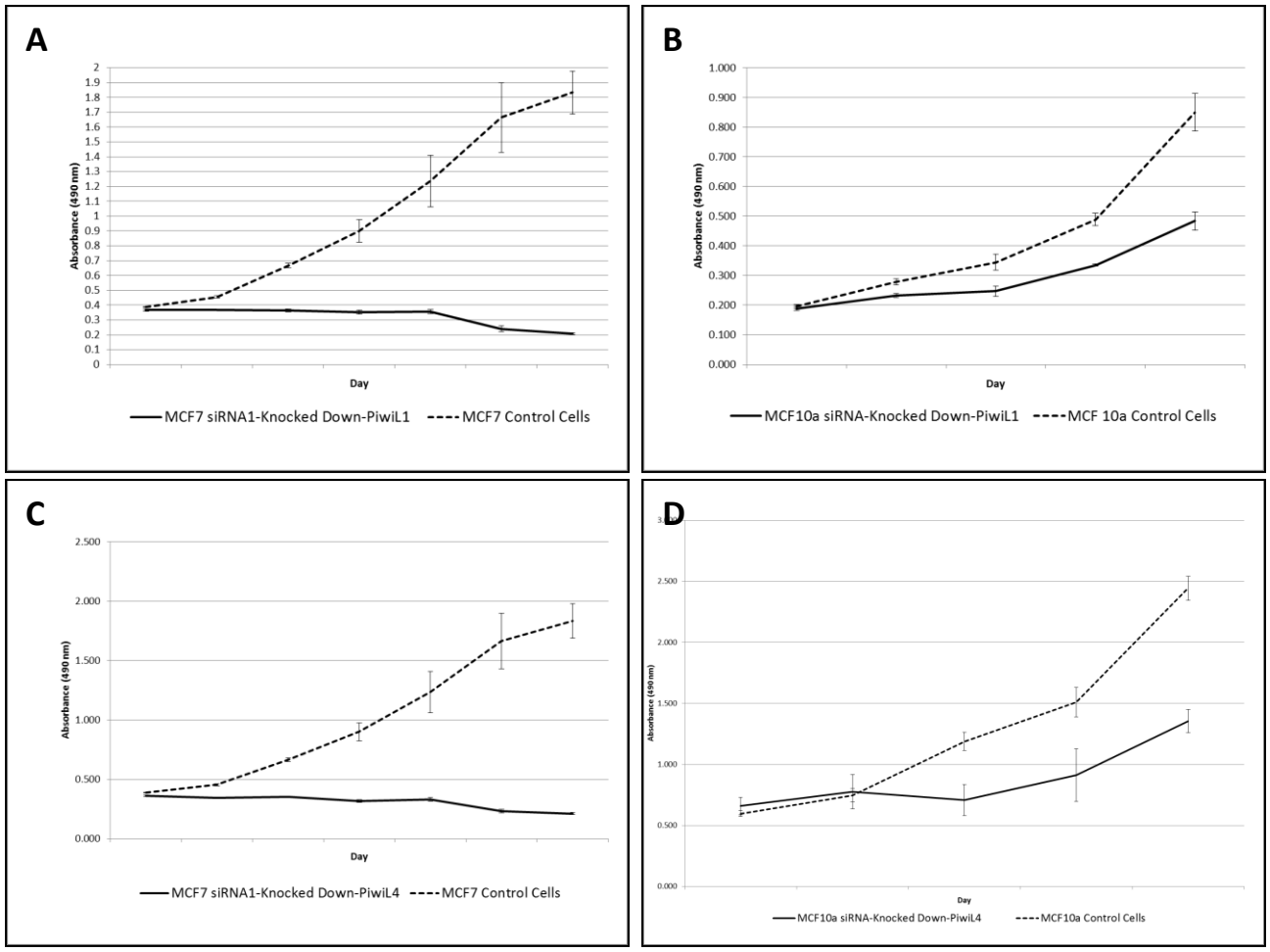


Figure 5 Cell proliferation rates of MCF7 and MCF10a between PIWIL1 knockdown group, PIWIL4 knockdown group and negative control. MCF7 and MCF10a cell proliferation rates were assessed at baseline, 24 hours, 48 hours, 72 hours, and 96 hours (and also at 120 hours and 144 hours for MCF7) following transfection with a PIWIL1/PIWIL4 targeting siRNA and a scrambled sequence negative control oligo. (A) Transfection with PIWIL1 targeting siRNA in MCF7 cells strongly slowed down cell proliferation compared to negative controls ($P < 0.0001$); (B) PIWIL1 knockdown only resulted in a moderate reduction in cell proliferation rate in MCF10a cells. (C) Transfection with PIWIL4 targeting siRNA in MCF7 cells greatly slowed down cell proliferation and even stopped growth altogether compared to negative controls ($P < 0.0001$); (D) PIWIL4 knockdown resulted in a moderate reduction in cell proliferation rate in MCF10a cells. Error bars represent standard deviations.

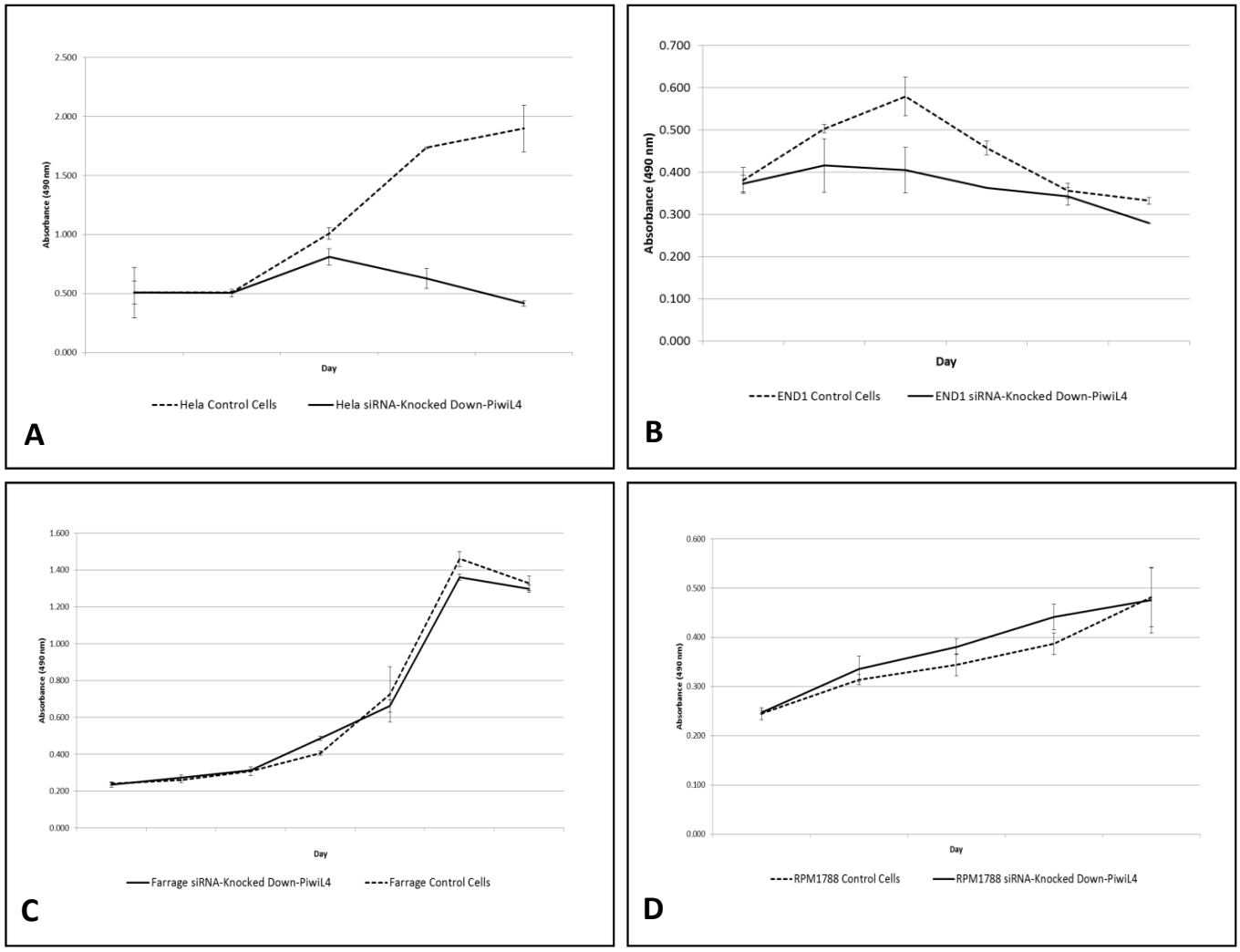


Figure 6 Cell proliferation rates between PIWIL4 knockdown group and negative control in cervical cancer and non-Hodgkin's lymphoma cell lines. Cell proliferation rates in cervical cancer and non-Hodgkin's lymphoma cell lines were assessed at baseline, 24 hours, 48 hours, 72 hours, and 96 hours following transfection with a PIWIL4 targeting siRNA and a scrambled sequence negative control oligo. (A) Transfection with PIWIL4 targeting siRNA in HeLa cells significantly slowed down cell proliferation compared to negative controls ($P < 0.0001$); (B) PIWIL4 knockdown resulted in a moderate reduction in cell proliferation rate in END1 cells where the difference did not reach statistical significance (C) Transfection with PIWIL4 targeting siRNA in Farrage cells resulted in no difference in cell proliferation rate compared to negative controls ($P > 0.05$); (D) Knocking down PIWIL4 resulted in no significant effect in cell proliferation rate in RPM1788 cells, as cells in the treatment group grew as quickly as those in the control group. Error bars represent standard deviations.

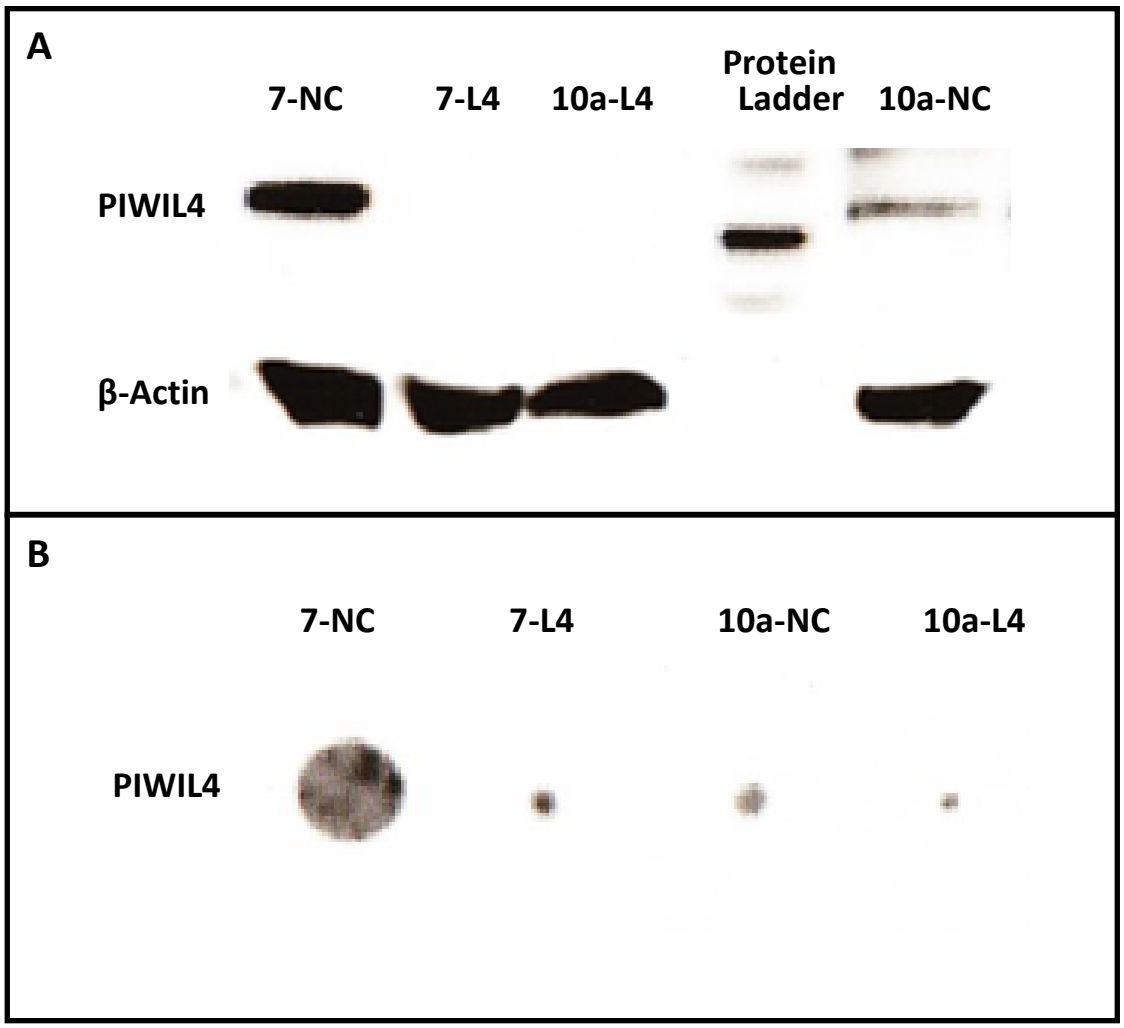


Figure 7 Western blotting and dot blotting for the confirmation of PIWIL4 knockdown in MCF7/MCF10a Cells. Whole proteins were extracted from the following four groups: **7-NC**: the negative control MCF7 cells; **7-L4**: MCF7 cells transfected with PIWIL4 targeting siRNA; **10a-NC**: the negative control MCF10a cells; **10a-L4**: MCF10a cells transfected with PIWIL4 targeting siRNA. The darkest band in the protein ladder corresponds to a protein size of 100KDa. (A) Western blotting: similar expression levels of β-actin were found in each of the four groups while no expression of PIWIL4 (protein size ~95KDa) was found in MCF7 cells and MCF10a cells transfected with PIWIL4 targeting siRNA. (B) Dot blotting results: the quantity of PIWIL4 proteins was larger in MCF7 negative control cells than that in MCF10a negative control cells. Both negative control groups had a much larger protein quantity of PIWIL4 compared to their respective siRNA transfected groups.

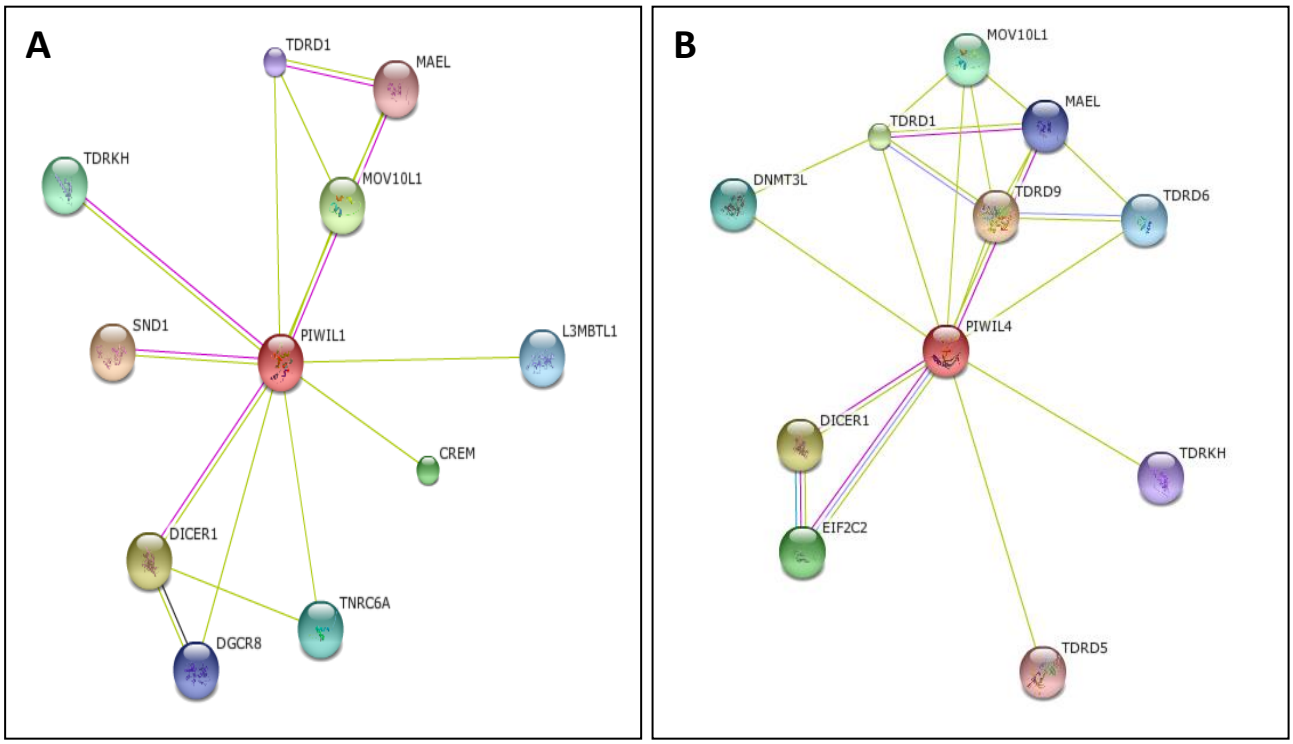


Figure 8 Known and predicted protein-protein interactions of PIWIL1 and PIWIL4

Potential interactions between selected PIWI proteins and functionally related proteins were constructed using the STRING platform (version 9.0.5). Different line colors represent the different types of evidence underlying the interactions. (A) Existing evidence suggested that PIWIL1 interacted with several key components in RNA-mediated gene silencing and transcriptional regulation, including DICER1, MOV10L1 and Tudor domain related proteins. (B) Proteins predicted to interact with PIWIL4 included RNA-mediated gene silencing factors like EIF2C2 and DICER1, Tudor domain related proteins like TDRD9, and regulatory factors of DNA methylation like DNMT3L.

INTRODUCTION

Globally, breast cancer is the most common invasive cancer in women, comprising 22.9% of invasive cancers in women and 16% of all female cancers. It is estimated that in 2013 alone, 232,340 women were diagnosed with and 39,620 women died of breast cancer in the United States[1]. Meanwhile, the prevalence of breast cancer is steadily rising, with the highest risks observed in industrialized nations[2]. However, less than half the risk in industrialized areas is attributable to changes in known risk factors for breast cancer[3].

Among these risk factors, shiftwork has been investigated by multiple epidemiologic studies and identified by IARC as 'probably carcinogenic to humans'. Although several findings have reported that people who work the night shift are at increased risk of developing breast cancer[4,5,6], the detailed mechanism by which shiftwork can trigger tumorigenesis remains poorly understood. Our previous study, drawing from subjects of the Danish "Diet, Cancer and Health" prospective cohort, demonstrated significant epigenetic associations between the CLOCK and CRY2 circadian genes and night shiftwork using a whole-genome methylation screening approach. Based on the same approach, we recently discovered a significant change in PIWI gene methylation among night shiftworkers within the same population, which may provide additional insight into the molecular relationship between breast carcinogenesis and night shiftwork.

PIWI proteins, which comprise a subclade of the Argonaute protein family, are known to play important roles in spermiogenesis[7], stem cell self-renewal[8], RNA silencing[9], and translational regulation in a multitude of organisms[10,11]. Recently, an increasing body of evidence has accumulated and suggested that PIWI proteins are aberrantly expressed in a

variety of cancers[12]. However, thus far, only one study has ventured to study the relationship between PIWI genes and breast cancer. Moreover, despite the discovery of their expression in human somatic cancers, research on PIWI proteins in cancer remains at an early stage[13], as very few studies have carefully examined the molecular mechanisms by which PIWI proteins contribute to tumorigenesis.

In the present study, methylation changes in PIWI genes were examined using whole-genome methylation analysis using subjects from the Danish shiftworker population, who have previously been reported to be at high risk for breast cancer[4]. In addition, we conducted expression analyses and *in vitro* functional tests to explore PIWI's potential role in breast tumorigenesis. Finally, we explored potential molecular mechanisms underlying the observed PIWI gene expression changes and the functional impact imparted by PIWI gene downregulation in breast cancer cells.

METHODS

Study Subjects and Shiftwork Exposure Assessment

As previously reported[14], study subjects were selected among the female participants who were recruited to the Danish "Diet, Cancer and Health" prospective cohort[15], established between December 1993 and May 1997. All female participants were born in Denmark, free of any cancer and aged between 50 and 64 years at time of invitation (now between 65- and 79- years-old). Information on food consumption, folate intake, other lifestyle factors (e.g., tobacco smoking, alcohol habits, sun exposure, physical activity, and

medical anamnesis), reproductive factors, education and occupation were collected in the baseline questionnaire. All collected biological samples were frozen and kept at -150°C. Blood DNA samples were available for methylation analysis.

Night work was defined as having worked fulltime starting from 7 pm or later and ending before 9 am in the next day. Telephone interviews were conducted to obtain information on night shiftwork status. In the current study, ten female long-term night shiftworkers (age: 54.8±3.6, years of shiftwork: 21.2±8.8) were selected as the shiftworker group. Ten female day workers (age: 54.0±3.3, years of shiftwork: 0), matched to subjects of the shiftworker group by age (± 2 years) and total folate intake (± 55 μg/day), were selected for comparison with the shiftwork group. Additional details on the study poopulation have been described previously[16].

Genome-Wide CpG Island Methylation Assay

Genomic DNA of 10 pairs of long-term night shiftworkers and day workers was isolated and purified from blood samples using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's protocol. These genomic DNA were then bisulfite treated using the EZ DNA Methylation Kit (Zymo research). 50 ng of genomic DNA from each participant in both shiftworker were submitted to Yale University's W.M. Keck Foundation Biotechnology Research Laboratory for genome-wide DNA methylation measurements using Illumina's (San Diego, CA) Infinium HumanMethylation27 array platform. Quantitative measurements of DNA methylation were determined for 27,578 CpG dinucleotides spanning 14,495 genes. Tested CpG sites include those located within the proximal promoter region of each gene, ranging from 0 to 1499 bp upstream of the

transcription start site (average ± SEM distance = 389 ± 34 bp). A methylation index (β) was used to estimate the methylation level of each CpG locus using the ratio of intensities between methylated and unmethylated cytosines, which is a continuous variable between 0 and 1. 0 corresponds to a completely unmethylated site, while 1 corresponds to a completely methylated site. The Illumina Custom Model, as implemented in the Illumina GenomeStudio software, was used to assess the statistical significance of methylation differences between night shiftworkers and day workers. To correct for the increased likelihood of false positive differences resulting from multiple testing, the Benjamini-Hochberg method was used to control for the False Discovery Rate (FDR), defined as the expected ratio of erroneous rejections of the null hypothesis to the total number of rejected hypotheses. Methylation differences at CpG sites were considered statistically significant at a FDR-corrected P-value (Q-value) <0.05.

Data mining of PIWI expression in breast cancer and other tumor types

A comprehensive search using the Oncomine online database (https://www.oncomine.org; accessed on November 29th, 2013) was performed to investigate whether PIWI gene expression are altered in breast cancer as well as other cancer types. Comparisons were made based on for expression array results involving tissues drawn from cancer patients and healthy controls. The filters used were: Gene: "PIWIL1", "PIWIL2", "PIWIL3", or "PIWIL4"; Analysis Type: "Cancer vs. Normal Analysis"; Caner Type: "Breast Cancer". The search returned a total of 36 analyses conducted in 12 unique studies for breast cancer using different array platforms (10 for PIWIL1, 9 for PIWIL2, 4 for PIWIL3 and 13 for PIWIL4 respectively). Further details regarding tissue collection and the

experimental protocol of each array are available in the Oncomine database, or from the original publications.

Cell culture and treatments

All experimental procedures were approved by the Institutional Review Board at Yale University and the National Cancer Institute. Human breast adenocarcinoma cells (MCF-7) (American Type Culture Collection, Manassas, VA) were maintained as monolayer cultures in 25 cm^2 polystyrene culture flasks (Falcon, Becton Dickinson BioScience, Le Pont de Claix, France) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA).

Human breast MCF10a cells were maintained as monolayer cultures in 25 cm^2 polystyrene culture flasks (Falcon, Becton Dickinson BioScience, Le Pont de Claix, France) in MEGM medium (American Type Culture Collection, Manassas, VA), 100 ng/ml cholera toxin (Sigma-Aldrich, St. Louis, MO), and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) .

Human HeLa cervical cancer cells (American Type Culture Collection, Manassas, VA) were maintained as monolayer cultures in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO). Cells were incubated at 37℃ in a humid atmosphere containing 5% CO_2 and subcultured every 2-3 days.

Human End1 cervical cells (American Type Culture Collection, Manassas, VA) were maintained as monolayer cultures in KSFM medium (Invitrogen, Carlsbad, CA)

supplemented with 0.1 human recombinant EGF (Invitrogen), and 0.05 mg/ml bovine pituitary extract (Invitrogen). Cells were incubated at 37℃ in a humid atmosphere containing 5% CO_2 and subcultured every 2-3 days.

Human Farage B-cell lymphoma and RPMI 1788 B cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% and 20% fetal bovine serum (Invitrogen), respectively. Cells were incubated at 37℃ in a humid atmosphere containing 5% CO_2 and subcultured every 2-3 days.

Pre-designed siRNA oligos (iDT) were used to knock down the expression of PIWIL1 and L4 in the cell lines described above. Transfection of the siRNA was performed using LipofectamineTM RNAiMAX (Invitrogen). AllStars Negative Control siRNA (Qiagen) was used to ensure minimal nonspecific effects on gene expression and phenotype as it exhibits no homology to any known mammalian gene. Each oligonucleotide was reverse-transfected in 6-well plates with 30,000 cells/well at a final concentration of 25 nM. After incubation at 37℃ and 5% CO_2 for 48 hr, total RNA samples were isolated.

RNA isolation and quantification

Isolation of total RNA from transfected cell lines was conducted using the miRNeasy Mini Kit (Qiagen), according to the protocol of the manufacturer for mammalian cells and tissues. On-column DNase digestion (Qiagen) was also conducted during the isolation of RNA. The quantification of isolated RNA was measured on an Epoch Microplate Spectrophotometer (Biotek, Winooski, VT). First-strand cDNA conversion

was performed using the AffinityScript Multi Temperature cDNA Synthesis Kit (Agilent Technologies).

PIWI Gene Expression Measurement by Quantitative RT-PCR

PIWI mRNAs expression levels were measured by quantitative real-time PCR performed in technical triplicate using the KAPA SYBR FAST Universal qPCR Kit (KAPA Biosystems) via a standard thermal cycling procedure on an ABI 7500 instrument (Applied Biosystems). RNA quantity was normalized using the HPRT housekeeping gene and quantified using the $2^{-\Delta\Delta Ct}$ method. The SYBR Green detection using the following primers:

PIWIL1	5'-AATAATATTTTCAGGAGGCTTTTG-3' 5'-AATCACCAACCTGTGACTTGG-3'
PIWIL2	5'-CCAGCACAAAGGGAGTCTGT-3' 5'-GCCTCTACCTGATGGAAGCA-3'
PIWIL3	5'-TTTAGAAGAACTTTCAAGCTGCTG-3' 5'-TCCAAACTGGTCCCATGACG-3'
PIWIL4	5'-TTCCCCAGCACAAATTATCC-3' 5'-ACGTCTGGGTGAAACAGGAC-3'

Cell Proliferation Assay

MCF7, MCF10a, Hela, End1, Farage, and RPMI 1788 cells were transfected with siRNA oligos targeting PIWIL1 or PIWIL4 and a scrambled sequence negative control in 96-well plates using the Lipofectamine RNAiMAX transfection reagent. Cell proliferation was measured in triplicate at baseline, 24 hours, 48 hours, 72 hours, and 96 hours (some groups extend to 144 hours) using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) kit (Promega Corporation, Madison, WI). 20 µL of MTS dye

were added to each well and allowed to incubate with transfected cells in a 37℃ CO_2 incubator for 2 hours. Following incubation, MTS-stained cells were quantified using an Epoch Microplate Spectrophotometer.

Western Blotting

Standard western blotting technique was used. Briefly, total protein lysate was collected from cells using RIPA buffer (Santa Cruz Biotechnology) and 30 ug were run on a 10% NuPAGE Bis-Tris gel (Life Technologies). PVDF membranes were incubated overnight at 4℃ with the following primary antibodies: PIWIL4 (1:1000, abcam 111714) or β-actin (1:2000 Santa Cruz Biotechnology).

Statistical Analysis

Statistical analyses were performed using the SAS statistical software, version 9.3 (SAS Institute). Student t-tests and one-way ANOVA were applied to calculate differences in PIWIL4 expression across different tumor types. A repeated-measures, mixed-design ANOVA model was used to assess whole curve differences in proliferation rate between transfection groups by measuring the interaction between transfection type and time.

RESULTS

PIWIL1 was significantly hypomethylated among long-term night shiftworkers

As previously reported[14], our whole-genome methylation analysis identified differential methylation between day workers and long-term shiftworkers at 5409 CpG sites, corresponding to 4752 genes, after adjusting for multiple comparisons. Among all 27,578 CpG sites analyzed, a total of 4 CpG sites were located in the PIWI gene regions, including 2 in PIWIL1 and 2 in PIWIL2, covering the proximate promoter (TSS1500) or the 5' UTR/TSS200 region. Compared to day workers, all 4 CpG sites showed decreased methylation in shiftworkers. Methylation indices for day and shiftworkers at each of these CpG sites are presented in **Figure 1**. Of these 4 hypomethylated CpG sites, the cg13861644 site covering the proximal promoter region of PIWIL1 showed a statistically significant difference between day and shiftworkers at $Q < 0.001$, with a decrease in methylation level among night shiftworkers greater than 10% (Δ=13.14%, $Q=2.32*10^{-6}$). These results demonstrated that both PIWIL1 and PIWIL2 were hypomethylated to different extents in night shiftworkers. As previously reported[4], the odds ratio for breast cancer among women who worked as night shiftworkers in this population was 1.5 (95%CI: 1.2 to 1.7) compared to day workers. Typically, hypomethylation that occurs at CpG sites in the promoter region is associated with gene activation. The significant decrease in methylation at cg13861644 in the PIWIL1 promoter might result in overexpression of PIWIL1 among night shiftworkers, which might in turn be associated with the increased breast cancer risk in this population. Further in-depth investigations using existing expression array data and molecular methods were used to explore whether PIWI genes have potential oncogenic roles in breast tumorigenesis.

The Oncomine Database Indicates Differential Expression of PIWI Genes in Breast Cancer Compared to Normal Breast Tissue

To investigate the role of PIWI genes in breast tumorigenesis, a comprehensive search of the Oncomine database was conducted. Searching for PIWI gene expression in "cancer vs. normal" tissues in the Oncomine database returned a total of 36 analyses from 12 unique studies in breast cancer. 5 out of the 36 analyses were identified as statistically significant with P-values < 0.05. A bubble plot was generated using -log10 transformed P-values and the fold change of PIWI gene expression in breast tumor versus normal breast tissues extracted from each analysis. The size of each circle is proportional to the sample size of the analysis it corresponds to (**Figure 2**). The plot indicates that PIWI gene expression is frequently altered in multiple breast tumor subtypes, including invasive ductal breast carcinoma, ductal breast carcinoma, invasive lobular breast carcinoma and fibroadenoma. Specifically, 8 out of the 10 analyses with PIWIL1 expression data exhibited increased PIWIL1 expression in breast tumor relative to normal breast tissues while only two showed decreased expression (**Figure 2A**). Among them, three analyses showed statistical significant ($P<0.05$) higher PIWIL1 expression ranging from 1.033- to 1.407-fold in breast tumor compared to normal tissues. Similarly, 6 out of the 9 analyses with PIWIL2 expression data exhibited increased PIWIL2 expression in breast tumor relative to normal breast tissues, while three showed decreased expression (**Figure 2B**). Only one analysis (Finak, invasive breast carcinoma) showed a statistical significant expression change ($p=6.3*10^{-6}$) with 1.307 times higher PIWIL2 expression in tumor compared to normal tissue. Interestingly, while analyses with relative small sample sizes ($\leq$100) tended to report higher PIWIL2 gene expression, studies with large sample

sizes (~1000) tended to reveal slightly decreased PIWIL2 expression in tumor versus normal tissue. Only 4 studies had expression data available for PIWIL3 and 3 out of the 4 analyses exhibited statistically non-significant decreased PIWIL3 expression in breast tumor relative to normal breast tissues while one showed highly statistically significant increased expression (fold change=2.85, $p=1.1*10^{-12}$) in tumor relative to normal tissue (**Figure 2C**). In addition, 7 out of the 13 analyses with PIWIL4 data exhibited increased PIWIL4 expression in breast tumor relative to normal tissues while 6 showed decreased expression (**Figure 2D**). While the analysis results of PIWIL4 were inconsistent across different studies, none of the analyses were statistically significant at p=0.05.

In conclusion, the results derived from the Oncomine database showed that the expression of PIWI genes is up-regulated in breast tumor vs. normal tissues in the majority of the analyses (22 out of 36). Moreover, all studies where expression differences achieved statistical significance revealed higher PIWI gene expression in breast tumor tissues. While most studies report up-regulated expression of PIWIL1 and PIWIL2 in breast cancer, the expression differences in PIWIL3 and PIWIL4 were inconsistent. *In vitro* experiments were needed and therefore performed to further explore the oncogenic role of PIWI genes in breast tumorigenesis.

PIWI Gene Expression is Upregulated in Multiple Cancer Cell Lines Compared to Normal Tissue Cells

To investigate the role of PIWI genes in breast tumorigenesis, we measured the relative expression levels of all four PIWI genes in MCF7 breast cancer and MCF10a normal breast cells. As shown in **Figure 3**, PIWI gene mRNA expression levels were all

significantly different between MCF7 and MCF10a cells. Consistent with our finding of PIWIL1 hypomethylation in the night shiftworkers, who may be at higher risk of breast cancer, the expression of PIWIL1 in MCF7 cells was 5.57 times higher compared to that in MCF10a cells ($p<0.01$). Similarly, PIWIL3 was 4.194 times overexpressed in MCF7 cells ($p<0.01$). More dramatic is the finding of a 15.6-fold increase in PIWIL4 expression in MCF7 cells ($p<0.01$), the result of which remained consistent through multiple repetitions of the experiment. Meanwhile, inconsistent with the Oncomine and methylation analysis, PIWIL2 was found to be 4.193 times underexpressed in MCF7 cells ($p<0.01$).

To investigate whether PIWIL1 and PIWIL4 had similar expression patterns in other cancer types, we also measured their relative mRNA expression levels cervical cancer (Hela) and normal cervical cells (End1), as well as non-Hodgkin's lymphoma (Farage) and normal B cells (RPM 1788).

As shown in **Figure 4**, the expression patterns of PIWIL1 and PIWIL4 in cervical cancer vs. normal cervical cells were similar to those found in our breast cell lines. However, in non-Hodgkin's lymphoma cell lines, while PIWIL1 was 3.59 times overexpressed compared to normal B cells, PIWIL4 was 28.14 times underexpressed, indicating that PIWIL4 might not be required for the growth of non-Hodgkin's lymphoma cells.

Upon considering all the evidence, the expression of PIWIL1 and PIWIL4 exhibited the most consistency across the various analyses, and were thus chosen as candidate genes for further functional analysis.

Knocking Down PIWIL1/PIWIL4 Decreases Breast Cancer Cell Proliferation Rate

As suggested by the findings of our analyses, we tested PIWIL1 and PIWIL4's potential role in cellular growth and proliferation using a MTS assay. Two sets of pre-designed siRNA oligos were used to knock down PIWIL1 and PIWIL4 in MCF7 breast cancer and MCF10a normal breast cell lines. As shown in **Figure 5A**, transfection with PIWIL1-targeting siRNA oligos significantly decreased MCF7 cell growth compared to the negative control ($P < 0.0001$). Moreover, the PIWIL1 knockdown had an immediate and obvious effect within 24 hours after transfection, and 98% of the cells died after 48 hours. In contrast, over 60% of MCF10a cells survived and maintained growth after 48 hours following transfection (**Figure 5B**). The results were consistent with our previous finding of a 5.57-fold increase in PIWIL1 expression in MCF7 breast cancer cells compared to MCF10a normal breast cells, indicating that PIWIL1 may be essential for the cellular proliferation in MCF7 cells while being less important for MCF10a cells.

A similar pattern was observed for PIWIL4, where MCF7 cells transfected with PIWIL4-targeting siRNA oligos had a statistically significant decrease in proliferation rate compared to negative control cells ($P < 0.0001$). The effect of knocking down PIWIL4 in MCF7 cells was also lethal, and almost all cells stopped growing within 24 hours, with an over 95% death rate within 48 hours (**Figure 5C**). In contrast, PIWIL4 knockdown only moderately affected MCF10a proliferation, and 50% of the cells remained alive and growing after 48 hours compared to the negative control ($P<0.05$) (**Figure 5D**). The dramatic decrease in cellular proliferation rate resulted from the knockdown of PIWIL4 in MCF7 cells was also consistent with our previous finding of a 15.9-fold increase in

PIWIL4 expression in MCF7 vs. MCF10a cells, which suggests that PIWIL4 may also play a much more important role in the proliferation of MCF7 compared to MCF10a cells.

To investigate whether PIWIL4 had a similar effect in other cancer types, we also conducted cell proliferation assays using our PIWIL4-targeting siRNA in cervical cancer cells as well as non-Hodgkin's lymphoma cells.

As shown in **Figure 6A**, transfection with PIWIL4-targeting siRNA oligos significantly decreased Hela cell growth compared to the negative control ($P < 0.0001$), and almost 75% of transfected Hela cells died after 96 hours. Meanwhile, transfected End1 normal cervical cells were only moderately affected at 24 hours after transfection (**Figure 6B**). The results were also consistent with our previous finding of a 3.96-fold increase in PIWIL4 expression in Hela cervical cancer vs. End1 normal cervical cells, which, again, suggests that PIWIL4 may be essential for the cellular growth and proliferation of Hela cells.

Interestingly, knocking down PIWIL4 in both Farage and RPM1788 cells did not result in altered cell proliferation rates compared to the negative controls. This, again, was also consistent with our previous finding of a 28.14-fold decrease in PIWIL4 expression in Farage NHL vs. RPMI 1788 normal B-cells, which suggests that PIWIL4 might not be essential for the cellular proliferation of Farage cells.

Confirmation of PIWIL4's Knockdown in the Functional Studies

Since the siRNA used to knockdown PIWIL4 targeted its 3'UTR region, silencing was likely to have taken place at the post-transcriptional level. Therefore, Western blotting

and dot blotting were conducted to further confirm the knockdown of PIWIL4 in previous functional studies, where the protein level of PIWIL4 in cells transfected with PIWIL4-targeting siRNA oligos and negative control cells were compared. As shown in **Figure 7**, the knockdown was confirmed in both MCF7 cells and MCF 10a cells transfected with PIWIL4-targeting siRNA oligos, indicating that the phenomenal impact observed in our functional analyses was likely the result of PIWIL4 knockdown.

DISCUSSION

As shiftwork has been defined as 'probably carcinogenic to humans' by IARC and a large percentage of the working population are engaged in shiftwork, the adverse health effects of shiftwork, which range from cardiovascular disease and cancer, have been widely investigated by many epidemiologic studies. However, few studies have ventured to study the molecular mechanisms underlying the connection between shiftwork and disease.

Using samples from the Danish "Diet, Cancer and Health" prospective cohort, our research group has previously reported that long-term exposure to shiftwork can alter epigenetic patterns [17] , and that there were statistically significant differences in methylation at many imprinted genes in shiftworkers compared to their day working counterparts[18]. The result of this study is consistent with these findings, and specifically show that exposure to shiftwork results in a significant decrease in the promoter methylation of PIWIL1. Although the molecular mechanisms underlying how long-term

night shiftwork may induce epigenetic alterations in cancer-related genes and increased breast cancer risk remain obscure, our findings of a potential oncogenic role for PIWIL1 and PIWIL4 in breast tumorigenesis may provide a possible missing link.

PIWI proteins, previously reported to be expressed predominantly in the germline, have recently been documented to have ectopic expressions in a variety of cancers. Specifically, PIWIL1 was first detected in seminomas[19] and later in pancreatic cancer[20], cervical cancer[21], colorectal cancer[22], liver cancer[23] and lung cancer[24]. Aberrant expression of PIWIL2 was reported in seminoma[25], breast cancer[26], cervical cancer[27], ovarian cancer[28], and gastric cancer[29]. PIWIL3 was only found to be expressed in gastric cancer and PIWIL4 was overexpressed in gastric cancer and cervical cancer [30]. Up to date, our study is the first to report the DNA hypomethylation of PIWIL1 in a shiftworkers, a population that has been observed to carry a higher risk of developing breast cancer. Moreover, we are also the first to observe overexpression of PIWIL1 and PIWIL4 in breast cancer cells.

Despite the accumulation of evidence of aberrant PIWI gene expression in cancer, studies attempting to elucidate the detailed molecular mechanism of PIWI proteins in tumorigenesis remain scarce[31]. Possible proposed mechanisms include: (1) PIWIL1-associated DNA methylation and cyclin-dependent kinase inhibitor (CDKI) silencing in sarcomas[32]; (2) PIWIL2-induced activation of antiapoptotic factor BCLX and STAT3 in a fibroblast cell line[26]; and (3) PIWIL4-induced promotion of cervical cancer cell growth and invasion via inhibition of apoptosis through the p53/p14ARF. So far, these studies represent the strongest evidence in support of a potential oncogenic role for PIWI genes

in breast tumorigenesis. In the present study, we used siRNA oligos to knockdown PIWIL1 and PIWIL4 and demonstrated that they were essential for the cellular growth and proliferation of MCF7 breast cancer cells. This finding is concurrent with our finding of PIWIL1 hypomethylation in night shiftworkers, who are at higher risk of breast cancer. The consistency of these disparate observations suggest that shiftwork may be able to trigger epigenetic alterations at potential oncogenic genes, including members of the PIWI family, which may in turn lead to the promotion of cancer cell growth and increased risk of breast cancer. Further analyses, including examinations of genes affected by PIWI gene knockdown, are required to further reveal the potential cancer related pathways PIWI genes may be involved in and identify downstream targets. Furthermore, as our observation of rapid breast cancer cell death following PIWIL1 and L4 knockdown is consistent with previously published data implicating them in cellular apoptosis, further functional analyses examining the role in apoptosis-related pathways are needed to attain a more comprehensive understanding of the impact of PIWI genes in breast cancer cell viability.

As shown in **Figure 8**, the prediction results derived using the STRING platform indicated that PIWI proteins likely interact with core RNA-mediated gene silencing and transcriptional regulatory factors. In addition, consistent with previous studies and our findings, PIWIL4 was also predicted to interact with DNMT3L, which is involved in the induction of *de novo* DNA methylation.

As gene expression in tumorigenesis is known to be controlled by small regulatory non-coding RNA, there have been numerous works exploring the role of small RNAs in

cancer. The role of miRNAs and their gene targets have been elucidated and characterized in various cancer types[13]. Similarly, as PIWI proteins bind to PIWI-interacting RNAs (piRNAs), they may also play a critical role in the regulation of gene expression in cancer tissues via the PIWI/piRNA pathway. However, in contrast to the voluminous amount of experimental and observational evidence elucidating the miRNA-cancer connection, little is known about the role of the PIWI/piRNA axis in the carcinogenic process. Aside from robust *in vivo* and *in vitro* models, more systematic and large-scale studies are thus needed to obtain a more comprehensive understanding of the role of PIWI proteins and piRNAs in the carcinogenic process.

As the knockdown of PIWIL1 and L4 greatly limited the growth of MCF7 breast cancer cells while exerting only a moderate effect on immortalized normal MCF10a breast cells, a potential therapeutic strategy might take advantage of the preferential effect of PIWI genes on breast tumor tissues. In fact, in our recent pilot animal study, direct injection with siRNA-targeting PIWIL4 via delivery reagents (*in vivo* Jet PEI) indeed significantly decreased tumor size with a 90% inhibition rate on mice planted with MCF7 cells, demonstrating consistency with our *in vitro* functional analyses. Again, additional studies with larger sample sizes are required to further elucidate the capacity of PIWI genes as therapeutic targets.

This study has several limitations. Because of the unavailability of breast tissue samples in the Danish prospective cohort, we were unable to investigate the potential correlation between PIWI methylation changes in peripheral blood leukocytes and expression changes in the breast experimentally. Another limitation is that the findings of the whole-

genome methylation analysis were based on a limited sample size, which in turn limited the statistical power by which interpretations could be made. As such, the results from the shiftwork methylation analysis should be interpreted carefully, as the degree to which they are generalizable cannot be known without further confirmation in an independent study population. Meanwhile, as mentioned above, additional functional studies, expression array measurements, and *in vivo* animal work are needed before we can claim PIWIL1 and PIWIL4 as important players in the breast carcinogenic process.

CONCLUSION

In summary, our findings provide the first evidence of a link between an epigenetic change in PIWI genes and night shiftwork. Moreover, the findings from our expression analyses and functional experiments both suggest that PIWIL1 and PIWIL4 may play an oncogenic role in breast tumorigenesis. Future mechanistic investigation into the function of PIWI genes is needed to further elucidate their role in breast carcinogenesis and to better inform the development of novel therapeutic strategies.

REFERENCES

1 Surveillance, Epidemiology and End Results (SEER) Program

2 Parkin DM et al., Cancer burden in the year 2000. The global picture. Eur J Cancer. 2001;37:S4-66.

3 Madigan MP, Ziegler RG, Benichou J, Byrne C, Hoover RN. Proportion of breast cancer cases in the United States explained by well-established risk factors. J Natl Cancer Inst. 1995;87:1681-5

4 Hansen J. Increased breast cancer risk among women who work predominantly at night. Epidemiology 2001;12: 74–77.

5 Stevens RG, Rea MS. Light in the built environment: Potential role of circadian disruption in endocrine disruption and breast cancer. Cancer Cause Control 2001;12: 279–287.

6 Davis S, Mirick DK, Stevens RG. Night shiftwork, light at night, and risk of breast cancer. J Natl Cancer Inst 2001;93:1557–1562.

7 Cox, D.N., Chao, A., Lin, H., 2000. Piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. Development 127, 503–514

8 Cox, D.N., Chao, A., Baker, J., Chang, L., Qiao, D., Lin, H., 1998. A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. Genes Dev. 12, 3715–3727.

9 Hutvagner, G., Simard, M.J., 2008. Argonaute proteins: key players in RNA silencing. Nat. Rev. Mol. Cell Biol. 9, 22–32.

10 Lin, H., Spradling, A.C., 1997. A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the Drosophila ovary. Development 124, 2463–2476.

11 Seto, A.G., Kingston, R.E., Lau, N.C., 2007. The coming of age for Piwi proteins. Mol. Cell 26, 603–609.

12 Suzuki, Ryusuke, Shozo Honda, and Yohei Kirino. "PIWI expression and function in cancer." Frontiers in genetics 3 (2012).

13 Ross, Robert J., Molly M. Weiner, and Haifan Lin. "PIWI proteins and PIWI-interacting RNAs in the soma." Nature 505.7483 (2014): 353-359.

14 Zhu, Yong, et al. "Epigenetic impact of long-term shiftwork: pilot evidence from circadian genes and whole-genome methylation analysis." Chronobiology international 28.10 (2011): 852-861.

15 Tjønneland, Anne, et al. "Study design, exposure variables, and socioeconomic determinants of participation in Diet, Cancer and Health: a population-based prospective cohort study of 57,053 men and women in Denmark." Scandinavian Journal of Public Health 35.4 (2007): 432-441.

16 Shi, Fengqin, et al. "Aberrant DNA methylation of miR‐219 promoter in long‐term night shiftworkers." Environmental and molecular mutagenesis 54.6 (2013): 406-413.

17 Bollati, Valentina, et al. "Epigenetic effects of shiftwork on blood DNA methylation." Chronobiology international 27.5 (2010): 1093-1104.

18 Jacobs, Daniel I., et al. "Methylation alterations at imprinted genes detected among long‐term shiftworkers." Environmental and molecular mutagenesis 54.2 (2013): 141-146.

19 Qiao, Dan, et al. "Molecular characterization of hiwi, a human member of the piwi gene family whose overexpression is correlated to seminomas." Oncogene 21.25 (2002): 3988.

20 Grochola, L. F., et al. "The stem cell-associated Hiwi gene in human adenocarcinoma of the pancreas: expression and risk of tumour-related death." British journal of cancer 99.7 (2008): 1083-1088.

21 Liu, Wen-Kang, Xiang-Yang Jiang, and Zhen-Xi Zhang. "Expression of PSCA, PIWIL1 and TBX2 and its correlation with HPV16 infection in formalin-fixed, paraffin-embedded cervical squamous cell carcinoma specimens." Archives of virology 155.5 (2010): 657-663.

22 Zeng, Yan, et al. "HIWI expression profile in cancer cells and its prognostic value for patients with colorectal cancer." Chinese Medical Journal-Beijing 124.14 (2011): 2144.

23 Zhao, Yi‐Ming, et al. "HIWI is associated with prognosis in patients with hepatocellular carcinoma after curative resection." Cancer 118.10 (2012): 2708-2717.

24 Liang, D., Y. Yang, and Y. Liu. "The role Hiwi gene in the maintenance of lung cancer stem cell populations." Neoplasma (2013).

25 Lee, J. H. et al. Stem-cell protein Piwil2 is widely expressed in tumors and inhibits apoptosis through activation of Stat3/Bcl-XL pathway. Hum. Mol. Genet. 15, 201–211 (2006).

26 Lee, Jae Ho, et al. "Pathways of proliferation and antiapoptosis driven in breast cancer stem cells by stem cell protein piwil2." Cancer research 70.11 (2010): 4569-4579.

27 Ye, Yin, et al. "Identification of Piwil2-like (PL2L) proteins that promote tumorigenesis." PLoS One 5.10 (2010): e13406.

28 Wang, Qi-En, et al. "Stem cell protein Piwil2 modulates chromatin modifications upon cisplatin treatment." Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 708.1 (2011): 59-68.

29 Wang, Yang, et al. "The PIWI protein acts as a predictive marker for human gastric cancer." International journal of clinical and experimental pathology 5.4 (2012): 315.

30 Su, Chang, et al. "PIWIL4 regulates cervical cancer cell line growth and is involved in down-regulating the expression of p14ARF and p53." FEBS letters 586.9 (2012): 1356-1362.

31 Siddiqi, Sara, and Igor Matushansky. "Piwis and piwi‐interacting RNAs in the epigenetics of cancer." Journal of cellular biochemistry 113.2 (2012): 373-380.

32 Siddiqi, Sara, Melissa Terry, and Igor Matushansky. "Hiwi mediated tumorigenesis is associated with DNA hypermethylation." PloS one 7.3 (2012): e33711.