


## Somatic MIWI2 Hinders Direct Lineage Reprogramming From Fibroblast to Hepatocyte

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### ABSTRACT

Remodeling of the gene regulatory network in cells is believed to be a prerequisite for their lineage reprogramming. However, its key regulatory factors are not yet elucidated. In this article, we investigate the role of PIWI proteins and provide evidence that one of them, MIWI2, is elicited during transdifferentiation of fibroblasts into hepatocyte-like cells. In coincidence with the peak expression of MIWI2, we identified the appearance of a unique intermediate epigenetic state characterized by a specific Piwi-interacting RNA (piRNA) profile consisting of 219 novel sequences. Knockout of MIWI2 greatly improved the formation of the induced hepatocytes, whereas overexpression of exogenous MIWI2 completely abolished the stimulated effect. A bioinformatics analysis of piRNA interaction network, followed by experimental validation, revealed the Notch signaling pathway as one of the immediate effectors of MIWI2. Altogether, our results show for the first time that temporal expression of MIWI2 contributes negatively to cell plasticity not only in germline, but also in developed cells, such as mouse fibroblasts. *STEM CELLS* 2019;37:803–812

### SIGNIFICANCE STATEMENT

PIWI proteins have long been identified as important factors in germline, but their role in somatic cells remains unclear, and only recently PIWI proteins have been linked to cancer development. This article uncovers a new somatic role for the PIWI proteins in mouse that regulate cell plasticity in a transdifferentiation model of fibroblast into hepatocyte-like cell. It also describes how Piwi-interacting RNA (piRNA) biogenesis respond to cell fate conversion, consistent with the proposed mechanism of piRNA acting as an “immune system” in cell.

### INTRODUCTION

Piwi-interacting RNAs (piRNAs), are small, single-stranded RNAs which are mainly expressed in germline and constitute the largest class of non-coding RNAs in eukaryotes [1–7]. Their biogenesis is unlike other small RNAs, and involves the ping-pong cycle mechanism [6, 8, 9]. piRNAs have an average length of 26–33 nucleotides and preferentially display a uridine at the 5' end and a 2'-O-methylated nucleotide at the 3' end [1]. DNA sequences encoding piRNA tends to cluster in chromosomes so that hundreds of different piRNAs can be translated at the same time [6, 10]. The proteins responsible for piRNA biogenesis and function are called PIWI proteins and are highly conserved across a wide spectrum of eukaryotes, ranging from sponges to humans [2, 5]. In many species there are three different PIWI genes for example, *Piwi*, *Aubergine* (*Aub*), and *Argonaute 3* (*Ago3*) in flies, or *Miwi* (Piwi-like 1, or *Piwil1*) *Mili* (Piwi-like RNA-mediated gene

silencing 2, or *Piwil2*), and *Miwi2* (Piwi-like RNA-mediated gene silencing4, or *Piwil4*) in mice [6, 11]. These three different proteins exert non-redundant functions. For example, in *Drosophila*, PIWI is expressed in the nucleus and is required for transposable elements (TEs) silencing, whereas AUB and AGO3 are necessary for post-transcriptional degradation of TE transcripts [11]. In the mouse, the two cytoplasmic PIWI proteins, MIWI and MILI are integral components of the machinery of primary piRNA processing, whereas MIWI2 is implicated in the secondary “ping-pong” cycle that catalyzes the maturation of the secondary piRNAs [8, 12]. MIWI has been implicated in LINE1 TE post-transcriptional silencing [12], whereas MILI and MIWI2 were shown to induce CpG DNA methylation of TE sequences in nucleus with MIWI2 being a direct recruiter of DNA methylation components [13–15].

In addition to its well-established roles in germlines, the PIWI-piRNA pathway has recently been found to exert diverse functions in a range

of somatic cells, especially in cancer cell lines [16]. Growing evidences support involvement of PIWI–piRNA pathway in cell regeneration and reprogramming. PIWI and piRNA activation was observed during pathophysiological liver regeneration in rat [17]. In *Podocoryne carnea*, the *Piwi* homolog, *Cniwi* was found to be highly expressed in lineage reprogramming of striated to smooth muscle cells [18]. However, in another study it was shown that all three PIWI proteins are dispensable for somatic development and reprogramming of fibroblasts into pluripotent stem cells in mouse [19].

Transdifferentiation, or lineage reprogramming, is a naturally occurring process in which a desired somatic cell is directly induced from another somatic cell without reversion to a pluripotent cell fate [20]. Following the discovery that is possible to generate induced pluripotent stem cell (iPSC) through reprogramming using four exogenous transcriptional factors [21], more experimental protocols using lineage-specific transcription factors have been developed to induce a variety of cell types (hepatocytes, neurons, pancreatic  $\beta$  cells, cardiomyocytes, etc.) without passing through pluripotent state [22–26]. Transdifferentiation involves activation of target genes in a time period that is comprised within hours to days. It is stable after the removal of the reprogramming factors and it does not require cell division [27, 28]. Transdifferentiation can be obtained starting from cells that share similar embryonic germ cell layer of the target cell or even across different germ cell layers [22, 25]. Two possible molecular mechanisms for cell reprogramming have been reported: one involving epigenetic modifications and the other caused by transient and stochastic interactions between transcription factors and chromatin architecture [22, 25, 27, 28]. The study of these exogenous regulatory factors offer important insight into the mechanism of direct lineage reprogramming, which is now believed to happen through the reconstitution of gene regulatory network (GRN) of target cell type [29, 30]. One potential mode-of-action is cell type conversion through an “on-target pioneer factor” [31]. This was illustrated in a mouse model of induced neuron (iN) cell conversion, in which *Ascl1* appeared to interact with its lineage-specific genomic targets, irrespective of the cellular epigenetic states, and initiate further interactions between other regulatory factors and GRN [29].

In this article, we investigated, for the first time, the expression and function of PIWI proteins in a cellular transdifferentiation model (tail-tip fibroblasts [TTFs] to induced hepatocytes [iHEPs] of the *p19<sup>Arf</sup>*<sup>-/-</sup> mice). The analysis of the expression level of the PIWI proteins during the transdifferentiation period, revealed that MIWI2 production, both at mRNA and protein levels, peaked around day 7 postinduction in coincidence with a major change in the piRNA expression pattern. By knocking out or knocking down *Miwi2* gene, we established that MIWI2 inhibits direct lineage conversion via the activation of a downstream Notch signaling. Finally, we showed that the expression of MIWI2 was transient and appeared to act as a “first responder” in somatic cells, indicating that MIWI2 plays a role as stabilizing factor in such cells as well.

## MATERIALS AND METHODS

Symbols of genes and proteins used herein follow the format of The Jackson Laboratory ([www.informatics.jax.org/mgihome/nomen](http://www.informatics.jax.org/mgihome/nomen)). Exogenous genes present in nonitalic style. PIWI refer to all Piwi-like proteins. Novel piRNAs emerged from deep

sequencing analysis were assigned a number arbitrarily, for example, *piR-000*.

### Conversion of Mouse Fibroblasts to Hepatocytes

Induction of hepatocyte-like cells (iHeps) from mouse fibroblasts was carried out using a modified version of the protocol reported by Huang et al. [25]. Briefly, the TTF cells of the *p19<sup>Arf</sup>*<sup>-/-</sup> or wild-type mice were isolated, expanded, and maintained in a Dulbecco's modified Eagle's medium/F12 (Gibco, Grand Island, NY) medium supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY). The lenti-viral particles of vectors (pWPI-psPAX2-pMD2.G, Addgene) containing only two transcription factor genes (i.e., *Hnf1a* and *Foxa3*) were transduced into the isolated TTF cells for 2 days. The resulting cells were inoculated in a modified Block's medium in a collagen-I coated dish/plate. In approximately 10–15 days, cells with epithelial characteristics began to emerge. Cells were then harvested and purified for functional determination.

To assess the role of Jag1/Notch2 pair in transdifferentiation process, 25 nM mouse Jagged 1 (Jag1) neutralizing antibody (Sigma, Steinheim, Germany) was added to the cell culture at day 2 postinduction. Fresh anti-Jag1 antibody was replenished during transdifferentiation.

### Cellular Storage of Glycogen

To assess the hepatic function of iHeps, the storage of glycogen in iHeps was stained by the Periodic Acid–Schiff base (PAS; Sigma–Aldrich, Steinheim, Germany). Cells were first fixed using the Carnoy's fluid and rinsed with running tap water. The periodic acid staining solution was then added to cover the cells for 5 minutes at room temperature. Finally, cells were rinsed with distilled water followed by incubation in the Schiff's reagent for 15 minutes at room temperature. Cells were counter-stained with Hematoxylin.

### Cellular Uptake of Low-Density-Lipoprotein

We assessed the epithelial property of iHeps by measuring if uptake of the acetylated low-density-lipoprotein (acLDL, Invitrogen, Eugene, OR, USA) could occur in iHeps. Cells were incubated in a culture containing 10  $\mu$ g/ml Dil (fluorophore) tagged acLDL at 37°C under 5% CO<sub>2</sub> for 4 hours. The resulting cells were rinsed with phosphate-buffered saline (pH 7.4) and cultured into a fresh media before detection. Fluorescence microscopy and FACS analyses were carried out. The excitation and emission wavelength of Dil are 561 nm and 575 nm, respectively.

### Detection of Albumin Secretion

AlphaLISA (PerkinElmer) assay was used to measure the amount of secreted albumin in culture media. Supernatant of culture was diluted and then mixed with the reagents of a commercial kit (PerkinElmer, Boston, MA). The fact that a long wavelength excitation (680 nm) on donor beads generates a short wavelength emission (615 nm) on acceptor beads in AlphaLISA warrants energy transfer without nonspecific fluorescence interference. Envision (PerkinElmer) was used to detect the signal.

### piRNA Profiling

Total RNA was extracted from triplicate cell samples using TRIzol (Invitrogen, Thermo, Carlsbad, CA). Next, small RNA libraries (<200 nt) were isolated and prepared. Next-generation-sequencing was carried out on an Illumina HiSeq2500 by

Genergy Biotechnology (Shanghai, China). The following criteria were adopted to assure data quality: (a) OD260/280 values ranging within 1.8–2.2; (b) sample concentrations greater than 500 ng/μl; (c) ratio of 28S RNA to 18S RNA greater than 1.5; and (d) RNA integrity number above 7. FastQC was applied to assure the quality of the postsequencing reads.

Approximately 1 million high quality clean reads were generated from each sample. Among them, we selected candidate piRNA sequences with a length between 26 and 33 nucleotides and excluded known sequences present in either Rfam (<http://rfam.xfam.org/>) or miRBase (<http://www.mirbase.org>). Reads that could be mapped to piRNABank (<http://pirnabank.ibab.ac.in/>) and GenBank (<http://www.ncbi.nlm.nih.gov>) were referred to as conserved piRNAs. piRNA predictor [32] and proTRAC [33] were used to identify potential novel piRNAs. The prediction for novel piRNAs follows principles such as typical length, clustered expression, biased bases at initial and position 10, reads distribution, and so on. Results with less than 10 reads were also eliminated to assure high quality. Finally, reads number of each piRNA was normalized to sample volume, evaluated as transcripts per million (TPM). piRNAs important to transdifferentiation were identified taking into account both TPM and relative fold changes ( $p < .01$ ). Z-score of each piRNA was calculated using the following equation:

$$z = \frac{x - \mu}{\sigma} \quad (1)$$

in which  $x$  represents TPM of piRNA;  $\mu$  represents the average TPM of the same piRNA in different testing samples; and  $\sigma$  represents standard deviation. Z-score measures the extent of variation of a particular piRNA along the time course of transdifferentiation. Hierarchical clustering (heatmap) of Z-scores was performed using ClustVis (<http://bit.cs.ut.ee/clustvis/>) [34] as shown in Figure 3B.

The above piRNA profiling was further validated using quantitative real-time PCR (qRT-PCR; SsoAdvanced, Bio-Rad). Total RNA was first poly-adenylated at 3' end and then reverse transcribed to cDNA by a universal primer (miScript II, Qiagen, Hilden, Germany). The sequence of forward primer was the same as that of the initial ~20 base pairs (bps) of a specific piRNA, whereas the reverse primer was a sequence based on the above universal reverse transcription primer. PCR products were validated by Sanger sequencing. The U6 RNA was used as an internal reference. The primers used are summarized in Supporting Information Table S1. Relative expression was analyzed using the  $2^{-\Delta\Delta Ct}$  method [35]. Triple biological replicates and triple technical replicates were used for each target.

### Gene Expression Analyses

Total RNA was extracted from cultured cells or liquid nitrogen grinded tissues using TRIzol (Invitrogen, Rockford, IL), and then reverse transcribed into cDNAs with the Maxima cDNA kit (Thermo, Vilnius, Lithuania). To determine the mRNA level of each target protein, the resulting cDNA was subject to qRT-PCR (SsoAdvanced, CFX Connect, Bio-Rad, Hercules, CA) analyses. Beta-actin (*Actb*) and glyceraldehyde-3-phosphate (*Gapdh*) were used as the internal references. Gene analysis was carried out as described in the previous section. The primers of piRNA processors and markers of cell type/status are listed in Supporting Information Tables S2 and S3, respectively.

### Western Blot Analyses

Cells were lysed in a RIPA lysis buffer (Pierce, Thermo) containing a cocktail of protease inhibitors (Pierce, Thermo, Rockford, IL). The resulting supernatant was harvested for Western blot detection. Anti-mPwi14 (anti-MIWI2) antibody (Santa Cruz, Dallas, TX), anti-Jag1 antibody (Abcam, Cambridge, UK), and anti-mNotch2 (Invitrogen, Taiwan, China) were used as the primary antibodies. The RIPA lysate of mouse testis tissue, which has a high level expression of the PIWI proteins, was used as a positive control for MIWI2.

### Immunostaining and Flow Cytometry

Before staining, cells were fixed by 4% polyformaldehyde and permeabilized by 0.1% Triton X-100 with 5% FBS. The primary antibodies against albumin/APC (R&D, Minneapolis, MN), CD26/PE (R&D, Minneapolis, MN, USA), E-cadherin/PE (R&D, Minneapolis, MN, USA), and MIWI2 (Abcam, Cambridge, UK) were used. The resulting cells were incubated at 4°C either overnight or 20 minutes and subject to immuno-cytochemistry (ICC) or FACS flow cytometry, respectively. The above cells were treated with a secondary anti-rabbit IgG conjugated with Alexa Fluor 405 for 1 hour at room temperature followed by characterization on a confocal microscopy, LSM 710 (Zeiss, Jena, Germany) and a FACS cytometry, Cytotflex S (Beckman Coulter, Indianapolis, IN).

### MIWI2 Knockout by CRISPR/Cas9

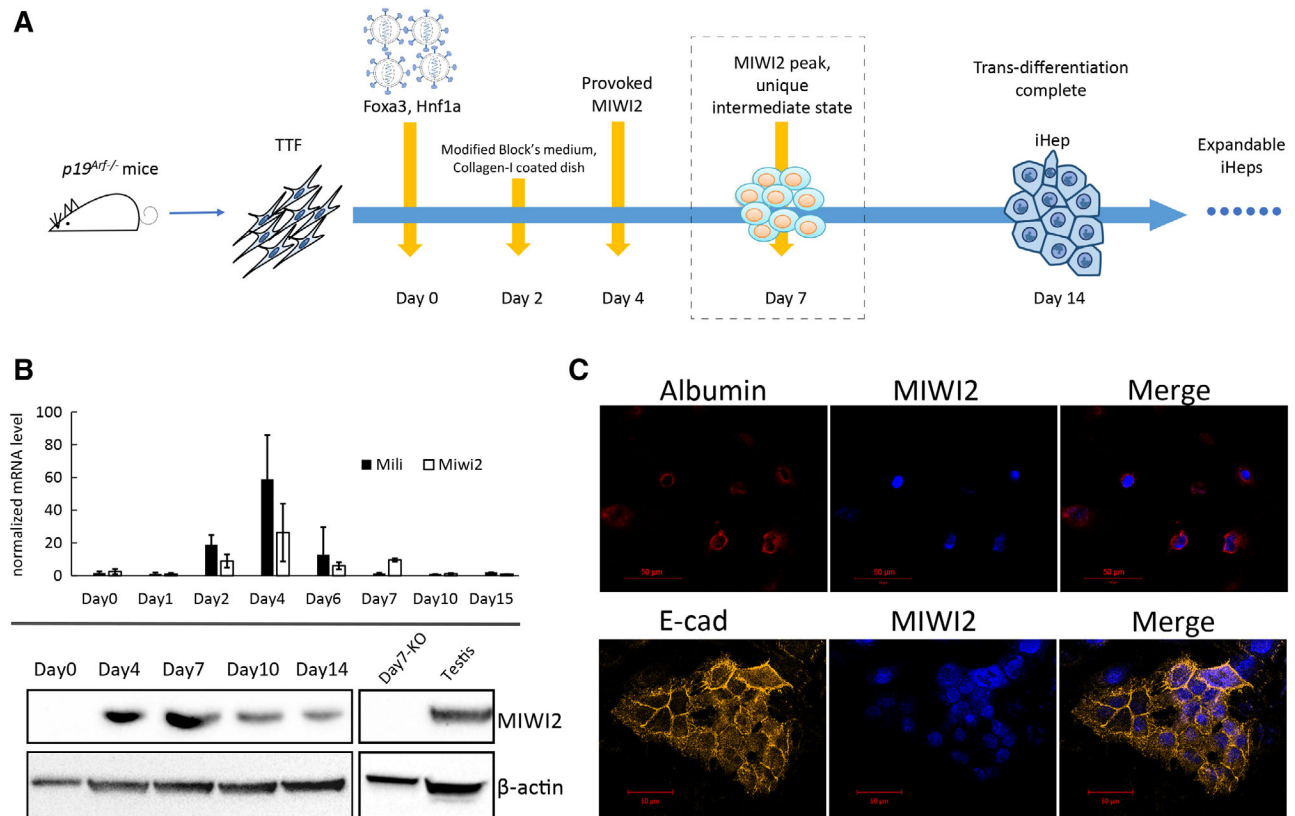
We designed three sgRNA sequences targeting different coding region of *Miwi2* according to the instruction published by Zhang Feng lab (<http://crispr.mit.edu/>). TTF cells were transfected using pSpCas9(BB)-2A-GFP and pGL3-U6-sgRNA (addgene) vectors containing single or combined *Miwi2* sgRNA sequences to assure the efficiency of the knockout. Transfected cells were sorted against GFP on FACS, and cultured for single clones. Clones were then selected by PCR amplification and DNA sequencing of the edited sequences in *Miwi2*. Western blot analysis was carried out to verify knockout of MIWI2 at protein level. Gene editing of *Miwi2* in TTFs was further validated by genomic sequencing.

### MIWI2 Knockdown by RNA Interference

Two pairs of siRNA sequences with 2'-O-methylated modification at 3' end and FAM labeling at 5' end, targeting different coding regions of *Miwi2*, were synthesized (GenePharma, Shanghai, China) and transfected into the Hnf1a and Foxa3 treated TTF cells by Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA) at a final concentration of 50 nM at day 2 postinduction. Western blot and qRT-PCR were used to identify cells that display more than 70% knockdown (KD) efficiency. For day 14 samples, to assure sustained gene suppression, cells were transfected for a second time at day 9 postinduction. A scrambled siRNA was used as negative control. Sequences of siRNA pairs for *Miwi2* and the scrambled siRNA control are GGAACCAAGUCUGGAUUUA/CACCUUAUCUGA CCUAAAU and UUCUCCGAACGUGUCACGU, respectively. These sequences all bear two thymine nucleotides overhang at the 3' end.

### MIWI2 Overexpression

The expression plasmid bearing *Miwi2* coding region sequence (NM\_177905.3) under a CMV promoter was transfected into TTF cells treated with Hnf1a and Foxa3 using Lipofectamine



**Figure 1.** Temporal activation of PIWI proteins during transdifferentiation. **(A):** Illustration of two transcription factors induced transdifferentiation with a hypothetical intermediate cell state. **(B):** Top: Transcript level of *Mili* and *Miwi2* by RT-PCR (expressed as a mean value  $\pm$  SD). Bottom: Protein level of MIWI2 detected by Western blot (mouse testis cell was used as a MIWI2 positive control). **(C):** Coexpression of MIWI2 (blue) and albumin (red) or E-cadherin (orange) at day 7 postinduction in nucleus. Scale bar represents 50  $\mu$ m.

3000 (Invitrogen, Carlsbad, CA) at day 2 postinduction according to the manufacturer's protocol. In the *Miwi2* rescue study, the expression plasmid containing *Miwi2* was transfected into the above *Miwi2* KD cells (48 hours after RNA interference [RNAi] transfection). For samples of day 14 postinduction, cells were transfected with the overexpression vector for a second time at day 9. For the rescue experiment, the second transfection was carried out at day 11 postinduction (48 hours after the second RNAi transfection). Overexpression of *Miwi2* was validated by quantitative RT-PCR and Western blot at day 7 and day 14 postinduction.

## RESULTS

### iHeps From Mouse Fibroblasts

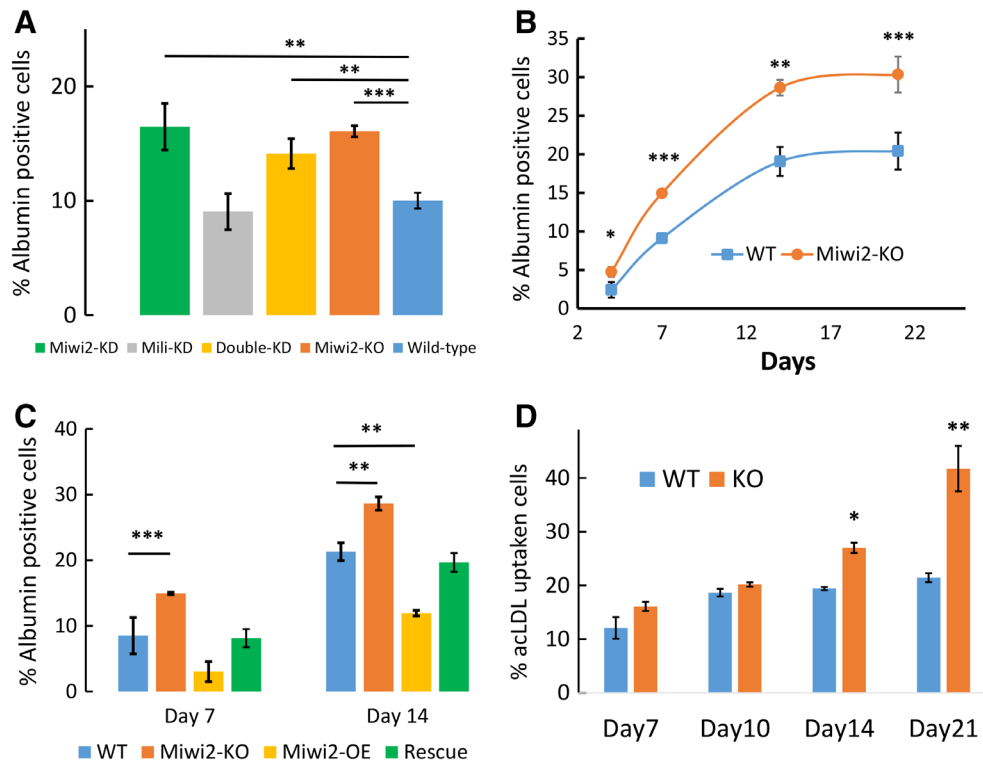
Transdifferentiation from mouse fibroblast to hepatocyte-like cell induced by three transcription factors has been already reported in the literature [25,36]. Here, we established an in vitro transdifferentiation model in which we iHeps from TTF cells of *p19<sup>Arf</sup>/-* mice after treatment with a combination of only two transcription factors (Foxa3 and Hnf1a) for 2 weeks (Fig. 1A). The resulting mouse cells showed a typical epithelial cell morphology and expressions of *Alb* (biomarker of hepatocytes) and *Ecad* (biomarker of epithelial cells; Supporting Information Fig. S1A). Approximately 20% of the resulting cells at day 14 expressed the albumin (Alb) marker as determined by flow cytometry (FACS; Supporting Information Fig. S1B). The

hepatic functions observed (lipid uptake and metabolism; Supporting Information Fig. S1C) and expression of hepatocyte specific genes (Supporting Information Fig. S1D) provided convincing evidence that these are iHep cells [25,36]. Furthermore, gene profiling was carried out for endoderm specific gene *Foxa2*, hepatic genes *Alb*, *Hnf4a*, *Cyp3a13*, and fibroblast enrichment gene *Thy1* throughout the lineage reprogramming process (Supporting Information Fig. S1D). Expressions of endodermic and hepatic specific genes were shown to begin at day 7 postinduction. At this time point, the gene expression pattern showed a mixture of mesodermal and endodermal genes, indicating an "intermediate cell type" in the lineage conversion from TTF cell to hepatocyte-like cell. Expressions of mesoderm gene *Brachyury (Bry)* and fibroblast specific gene *Thy1* appeared to decrease gradually during the transdifferentiation. No expression of pluripotent genes (*Oct4* and *Nanog*) or the ectoderm gene *Pax6* was detected along the process (Supporting Information Fig. S2).

### Temporal Expression of Piwi Proteins in Transdifferentiation

Quantitative RT-PCR analyses showed that the genes corresponding to MIWI2 and MILL, but not the *Miwi* gene, were significantly upregulated in the early stage of cell-to-cell conversion (days 4–7 postinduction), comparing to those in TTF and in iHep cells (Fig. 1B; top). The mRNA levels of corresponding piRNA processor proteins such as Hen1, Meal, Asz1, Prmt5, Tdrd1, and Tdrd6, were next analyzed, and elevated gene expression for all these





**Figure 2.** MIWI2 negatively regulated transdifferentiation from TTF into iHep. **(A):** *Miwi2* knockout (Miwi2-KO) or knockdown (Miwi2-KD) but not *Mili* knockdown (Mili-KD) significantly increased albumin positive cells at day 7 postinduction. **(B):** Miwi2-KO led to elevation of albumin positive cells throughout transdifferentiation. **(C):** Miwi2-KO increased albumin positive cells whereas overexpression (Miwi2-OE) or rescue (rescue) of *Miwi2* decreased albumin positive cells at day 7 and day 14 postinduction. **(D):** Miwi2-KO enhanced ability of acetylated lipoprotein uptake. Abbreviation: WT, wild-type cells. Data are represented as mean value  $\pm$  SD; \*,  $p < .05$ ; \*\*,  $p < .01$ ; t test.

proteins was observed at day 4 postinduction (Supporting Information Fig. S3). The two genes that displayed the highest expression were *Hen1*, a gene encoding the methyltransferase mediating piRNA methylation [37] and *Meal*, a gene encoding a key regulator in assembling/shuttling PIWI-piRNA complexes [38]. *Hen1*, in particular, showed sustained expression until day 7 (Supporting Information Fig. S3). The temporary increase in the expression of MIWI2 was confirmed also at the protein level by Western blot (Fig. 1B; bottom) and immunostaining (Supporting Information Fig. S4). The expression of MIWI2 peaked at day 7 postinduction but stayed under detection limit in TTF (day 0) and iHeps (day 14; Fig. 1B; bottom). A nearly identical expression pattern was observed in analogous experiment on *p19<sup>Arf</sup>/+* wild-type cells (Supporting Information Fig. S5) suggesting MIWI2 expression is not linked to *p19* deletion. No expression of the MILI protein was detected throughout the process of transdifferentiation; however, we cannot rule out the possibility that this is caused by the low sensitivity of MILI protein specific antibodies. Furthermore, immunostaining studies demonstrated that MIWI2 protein was coexpressed in the transition cells expressing albumin and E-cadherin (Fig. 1C) at day 7. Furthermore, MIWI2 was expressed mainly in nucleus similarly to what was observed in germline cells [12, 13], indicating a possible DNA methylation effector role for MIWI2 in the regulation of cell fate conversion.

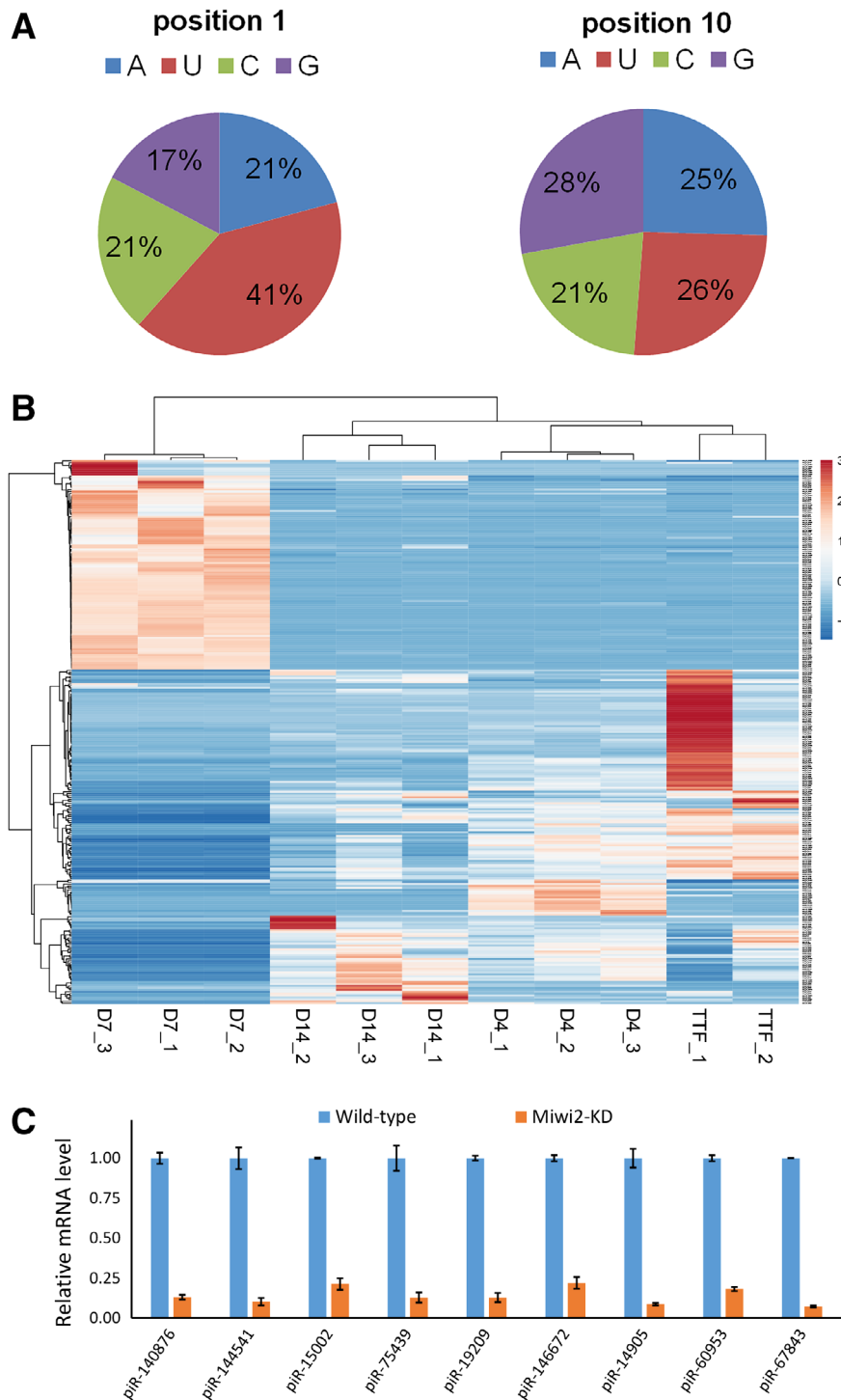
### MIWI2 Negatively Regulated Transdifferentiation From TTF to iHeps

In order to investigate the effect of transient expression of MIWI2 protein, we applied CRISPR/Cas9 and siRNA methods to

generate cell lines with MIWI2 knockout (KO), and MIWI2 and/or MILI KD, respectively. MIWI2 KD or KO alone increased the production of ALB positive cells, whereas a similar experiment, in which MILI was knocked down, did not produce any change in ALB production (Fig. 2A). FACS experiments showed that the enhancement effect observed after KD or KO of MIWI2 lasted throughout the whole transdifferentiation process (Fig. 2B). Moreover, MIWI2 KO significantly increased the expression of several hepatic genes such as *Hnf4a*, *Ttr* and so on at day 7, day 14, and day 21 (Supporting Information Fig. S6). On the other hand, overexpression of MIWI2, appeared to impede the switch from fibroblast into iHeps at day 7 and day 14 postinduction. On a similar note, expression of the exogenous *Miwi2* gene was sufficient to rescue the MIWI2 KO effect (Fig. 2C). Importantly, iHeps with *Miwi2* KO showed greatly elevated acLDL uptake activity (Fig. 2D). Albumin secretion was also detected in the supernatant of cultures containing MIWI2 KO and wild-type iHep cells (Supporting Information Fig. S7). On the contrary, knocking down *Mili* gene alone resulted in little difference comparing to the wild-type (Fig. 2A). Therefore MIWI2, not MILI and MIWI, is the key regulatory PIWI protein in response to the direct lineage reprogramming from fibroblast to iHep.

### Expression Patterns of piRNA During Transdifferentiation

By using small RNA deep-sequencing, we carried out piRNA profiling in cell samples at day 0 (TTF stage), day 4, day 7, and day 14 (iHep stage) postinduction (Fig. 3). Small RNAs with length from 26 to 33 nucleotides were sequenced and analyzed. Sequences



**Figure 3.** piRNA profiling. **(A):** Distribution of nucleotide usage for novel piRNAs at position 1 and position 10. **(B):** Heatmap of Z-scores of piRNA transcripts at different stages of transdifferentiation. At day 7, it showed a dramatically opposite Z-score pattern comparing to that of tail-tip fibroblast (day 0), day 4, and iHep (day 14). **(C):** Quantitative RT-PCR validation of differentially expressed piRNAs in wild-type versus *Miwi2* knockdown cells. Data are represented as mean value  $\pm$  SD.

showing an apparent U bias at position 1 and an equal nucleotide probability at position 10 from 5' end (Fig. 3A) were identified as primary piRNAs [2, 39–41]. Most piRNA sequences identified were novel and exhibited minimal overlaps with known germline piRNA clusters in mouse testis (piRNABank, <http://pirnabank.ibab.ac.in/>). It is noted that the novel piRNAs discovered in our experiments

greatly exceed the number of known mouse piRNAs, for example 109 out of 114 piRNAs in TTFs, 281 out of 298 piRNAs in the converting cells at day 4 postinduction, and 298 out of 316 piRNAs in iHeps, to the best of our knowledge, are not mentioned in the literature. At day 7 postinduction, which coincides with the peak expression of MIWI2, the number of novel and total piRNAs also

**Table 1.** Potential signaling pathways recognized by MIWI2 induced piRNAs

Pathway id	Pathway name	Pathway class	p value	FDR
mmu04512	ECM-receptor interaction	Signaling molecules and interaction	1.22E-11	7.80E-10
mmu04020	Calcium signaling pathway	Signal transduction	1.80E-11	9.22E-10
mmu04151	PI3K-Akt signaling pathway	Signal transduction	8.19E-11	3.50E-09
mmu04010	MAPK signaling pathway	Signal transduction	1.67E-09	4.74E-08
mmu04310	Wnt signaling pathway	Signal transduction	2.77E-08	5.87E-07
mmu04012	ErbB signaling pathway	Signal transduction	6.04E-08	1.10E-06
mmu04070	Phosphatidylinositol signaling system	Signal transduction	1.41E-07	2.01E-06
mmu04390	Hippo signaling pathway	Signal transduction	2.17E-07	2.41E-06
mmu04340	Hedgehog signaling pathway	Signal transduction	4.15E-07	4.25E-06
mmu04330	Notch signaling pathway	Signal transduction	6.14E-05	3.27E-04
mmu04370	VEGF signaling pathway	Signal transduction	6.89E-05	3.60E-04

Abbreviation: FDR, false discovery rate value (adjusted *p*-value).

peaked at 409 and 419, respectively, consistent with a key role of MIWI2 in the biogenesis of piRNAs during transcription factor induced cell fate conversion.

To study the link between piRNA biogenesis and lineage reprogramming, we selected 219 piRNA sequences that showed significant differential expression comparing to samples at day 0 (at least twofold differences with adjusted *p* values less than .001). Almost all of such sequences (204 over 219) were expressed at day 7, whereas at day 4 and day 14 their counts were 20 and 17, respectively (meaning that 22 sequences were found in more than one condition). The fact that nearly half of the newly synthesized piRNAs at day 7 (204 versus 419) showed differential expressions, and all are novel, suggests a strong correlation between piRNA biogenesis and MIWI2 production. Consistently with this finding, hierarchical clustering of Z-scores of these piRNAs showed a distinct piRNA expression pattern switch, especially in the cells at day 7 post-induction (Fig. 3B).

To test if piRNA biogenesis is mediated by MIWI2 in transdifferentiation, we quantitatively compared the expression level of nine piRNAs showing the highest differential expression at day 7 in wild-type and MIWI2 KD cells using quantitative RT-PCR. Our results, as expected, showed that MIWI2 KD resulted in a significant reduction (<20%) of all the tested genes (Fig. 3C).

### Signaling Pathways Associated With Cell Fate Conversion

PIWI protein interacting RNAs (piRNA) are known to silence transposons or genes at transcriptional or post-transcriptional level [2, 12–14, 42, 43], and both processes are sequence specific. Thus, we examined piRNAs that displayed increased expression during transdifferentiation and their specific targeting sequences of important biological pathways (see Materials and Methods). KEGG analysis revealed a total of 52 possible target pathway clusters, 11 of which being signaling pathways, essential to cell growth, proliferation, cell fate determination, and transcription regulation (Table 1, targets used for the analysis are listed in Supporting Information Table S4).

Among them, Notch signaling pathway has been known to play a role in lineage conversion in vivo. For example, inhibition of Notch signaling resulted in cell lineage conversion from club cells to ciliated cells in mouse [44].

### MIWI2 Inhibits Transdifferentiation by Activating Notch2-Jag1 Pathway

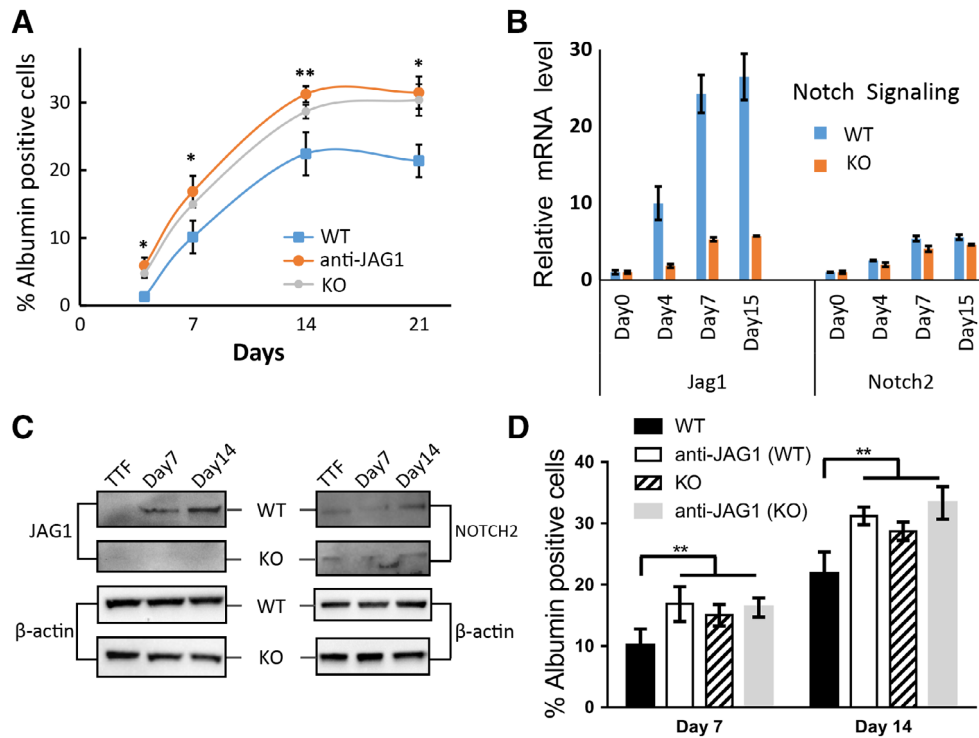
Despite the “bell shaped” curve of MIWI2 expression, the time course of transdifferentiation showed an initial exponential

phase followed by a saturated steady phase (Fig. 2B). MIWI2 KO cells showed an increase in both initiation rate and conversion efficiency of transdifferentiation (Fig. 4B). The elevation in the late steady phase of transdifferentiation (days 14–21) indicated that one or more effector pathways downstream of MIWI2 could have been activated. Ten representative genes of Notch pathway were selected and characterized using quantitative RT-PCR (Supporting Information Table S5). The Notch receptor ligand, *Jag1*, was the only member significantly upregulated by MIWI2 during the conversion (Fig. 4B, 4C). To understand the role of *Jag1*, a commercial *Jag1* neutralizing antibody was used, and the transdifferentiation experiments were carried out in both wild-type and MIWI2-KO TTFs. Our studies showed that during transdifferentiation, the *Jag1* expressions at different stages (Fig. 4A) matched the time course in the presence of the *Jag1* antibody (Fig. 4C) completely, consistent with *Jag1* as a downstream effector of MIWI2, which was activated and sustainably expressed throughout transdifferentiation. The fact that MIWI2-KO showed no apparent effect on *Jag1* inhibition (Fig. 4D) further confirmed the hypothesis.

## DISCUSSION

PIWI protein was first discovered in *Drosophila melanogaster* and much of the research so far, has been focused on the role of PIWI-piRNA complex in germline [5, 6]. The germline piRNAs are believed to participate primarily in the TEs silencing and gene regulations at epigenetic and post-transcriptional levels [2, 43, 45]. Nevertheless, increasing evidence of the presence of PIWI-piRNA in somatic cells prompts the interest of understanding PIWI signaling and its function in various physiological and pathological processes of adult cells [16]. As a naturally occurring process, transdifferentiation or direct lineage reprogramming alters cell fates between developmentally distant cell types. Various strategies using lineage-specific transcription factors, epigenetic regulators, microRNAs (miRNAs), small molecules, and so on have been explored to establish an efficient lineage reprogramming system [30].

In an attempt to correlate the function of PIWI and transdifferentiation, we examined the underlying regulatory mechanism in a direct reprogramming model of iHeps using the TTF from a *p19<sup>Arf</sup>-/-* mouse with two transcription factors, *Foxa3*, and *Hnf1a*. During the cell fate conversion, temporal expression of MIWI2 was observed at both mRNA and protein levels and shown dynamic and time-dependent progression.



**Figure 4.** MIWI2-dependent regulation of transdifferentiation by NOTCH signaling. **(A):** Blocking of JAG1 mediated NOTCH signaling by JAG1 neutralizing antibody (anti-JAG1) in WT cells photocopied *Miwi2* KO in albumin expression. **(B):** Gene expression of JAG1 and NOTCH2 during transdifferentiation in wild-type (WT) and *Miwi2* knockdown (KD) cells. **(C):** Protein expression of JAG1 (ligand) and NOTCH2 (receptor) during transdifferentiation in WT and *Miwi2* knockout (KO) cells. **(D):** Effect of Jag1 neutralizing antibody (anti-JAG1) on transdifferentiation of WT and *Miwi2*-KO cells. Data are represented as mean value  $\pm$  SD; \*,  $p < .05$ ; \*\*,  $p < .01$ ;  $t$  test.

Small RNA sequencing identified 219 novel mammalian piRNAs and a characteristic transient piRNA profile representing a unique intermediate epigenetic state along transdifferentiation. KD of MIWI2 in mouse fibroblasts reversed the upregulation of many piRNAs and greatly improved the formation of hepatocyte-like cells, whereas overexpression of exogenous MIWI2 completely abolished the stimulated effect indicative that MIWI2-piRNA signaling contributes negatively to the plasticity of mouse fibroblasts. Our study also revealed that activation of MIWI2 could elicit Notch signaling pathway as a downstream effector for the regulation. The observed MIWI2-dependent elevation of *Jag1* in Notch signaling over the course of iHep conversion strongly indicates that regulation of signaling pathways is a plausible mechanism for MIWI2's counteracting effect. This is consistent with previous studies showing that small molecule regulators induce lineage conversion through modulating various signaling pathways [46–49]. The experimental observation that disruption of the *Jag1*–*Notch2* signaling induced a direct conversion of club cells to ciliated cells in the lung of adult mice further strengthens our conclusion [44]. The transient expression of MIWI2 protein most likely induces a global epigenetic shift via biogenesis of novel piRNAs to target pre-existing signaling pathways related to lineage reprogramming.

#### CONCLUSION

Our current study reveals an important regulatory role of the somatic PIWI protein and piRNA in lineage reprogramming and provides a general framework to systematically investigate the

mechanisms governing the cell-to-cell conversion. The inhibitory role of MIWI2 we observed during the lineage conversion is consistent with its epigenetic function in maintaining genome integrity. iHep conversion appeared to adopt a similar hierarchical mechanism as the one proposed for iN conversion [29], and the temporal MIWI2 induction during conversion coincides with a distinct intermediate state of cell. It is striking that the larger number of novel piRNAs expression was observed at the intermediate state when MIWI2 expression peaked, in response to an “abnormal” alteration of cell epigenetic status. Analyses of the potential targets of these novel piRNAs yielded more than 50 pathways, 11 of which likely contribute to the lineage conversion. Considering the unique properties of piRNAs in achieving “self” and “nonself” recognition [50] and recent discovery of their regulatory roles in tumorigenesis [51–55], it is tempting to suggest that the described working model of PIWI–piRNA regulation could be a more general phenomenon happening in various cell fate conversions, including carcinogenesis.

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#### AUTHOR CONTRIBUTIONS

G.Y. and J.W.: conception and design, supervision of research, financial funding, manuscript writing; X.S.: conception and design, methods set up and performance of experiments, manuscript writing; Z.X.: methods set up, design and performance of experiments; F.Z.: bioinformatics analysis, financial funding, manuscript writing; W.W.: bioinformatics analysis; Y.W., Y.L., N.W., and Y.K.: performance of experiments; M.D., J.D.: financial funding.

#### DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

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#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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