PiRNA MW557525 Promotes the Vitality and Pluripotency of Piwil2-iCSCs by Regulating NOP56.

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Novel piRNA MW557525 regulates the growth of Piwil2-iCSCs and maintains their stem cell pluripotency

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[Abstract]

**Background:** CSCs play an important role in tumor development. Some studies have demonstrated that piRNAs participate in the progression of various cancers. However, the detailed function of piRNAs in CSCs requires further investigation. This study aimed to investigate the significance of some piRNAs in Piwil2-iCSCs. **Methods and Results:** Differentially expressed piRNAs in Piwil2-iCSCs were screened by high-throughput sequencing. Target genes were predicted by the miRanda algorithm and subjected to GO and KEGG analysis. One of the differential piRNAs, novel piRNA MW557525, was transfected and its target gene *NOP56* was silenced in Piwil2-iCSCs, respectively. RT-qPCR, western blot and dual luciferase reporter assay were used to investigate the interaction of piRNA MW557525 and *NOP56*. We identified the effect of piRNA MW557525 and *NOP56* knockdown on cell proliferation, migration, invasion, and apoptosis via CCK-8, transwell assay, and flow cytometry. The expressions of *CD24*, *CD133*, *KLF4*, and *SOX2* were detected via WB. The results showed that piRNA MW557525 was negatively correlated with *NOP56*, and it promoted the proliferation, migration, invasion, and inhibited apoptosis in Piwil2-iCSCs, and it also promoted the expressions of *CD24*, *CD133*, *KLF4*, and *SOX2*, while *NOP56* showed the opposite effect. **Conclusions:** These findings
suggested that novel piRNA MW557525 might be a novel therapeutic
target in Piwil2-iCSCs.

[Keywords] Piwil2-iCSCs; piRNA; NOP56; Proliferation; Migration;
Invasion; Apoptosis

[Statements and Declarations]

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interests.
1. Introduction

Cancer is a major disease threatening human health. At present, the global cancer burden is continuing to increase. It is estimated that by 2040, the incidence and mortality of cancer may double\cite{1}. The main methods of cancer treatment are surgical resection, combined with radiotherapy and chemotherapy. Traditional cancer treatment can only kill cancer cells with limited proliferation capacity, resulting in a decrease in tumor volume, but there are still a small number of cancer stem cells (CSCs)\cite{2}. The CSC hypothesis holds that there are a small number of cells (CSCs) in tumor tissues that have the ability to initiate and spread tumors for a long time\cite{3,4}. After a period of proliferation and differentiation, CSCs can form new tumors under the nourishment of their niche, leading to tumor reconstruction\cite{5}. Therefore, the treatment of CSCs may be an effective way to eradicate cancer, but CSCs themselves are resistant to a variety of therapies\cite{6}. Research on CSCs has mainly focused on finding CSCs in tumors and screening their markers. Studies have shown that Cluster of Differentiation 24 and 44 (CD24 and CD44) are usually associated with skin malignant melanoma and breast cancer\cite{7,8}, while Cluster of Differentiation 13 (CD133) is usually associated with bladder cancer and non-small cell lung cancer\cite{9,10}. Studies have also shown that the specific expression of miRNAs and piRNAs are specific markers for identifying CSCs\cite{11,12}.
Piwi–interacting RNAs (piRNAs) are type of single-stranded non-coding small RNA with a length of 24–31 nt; they interact with PIWI proteins to maintain the stability of key conserved genes in germ cells\cite{13}. PiRNAs and PIWI proteins, such as Piwi-like protein 2 and 4 (PIWIL2 and PIWIL4), have been shown to be differentially expressed in breast cancer, pancreatic cancer, liver cancer, lung cancer, gastric cancer, and other solid tumors\cite{14-16}, while the expression level and distribution of piRNAs vary with tumor types\cite{13, 17, 18}. Due to the resistance of CSCs to radiotherapy and chemotherapy, current tumor research is mainly focused on CSCs, while piRNAs have been less studied in tumor cells, especially in CSCs. At present, CSCs are mainly obtained by separating stem cells from solid tumor tissues, but there are still considerable difficulties in the extraction and culture of CSCs. The research group established a tumor-like stem cell model through PIWIL2 reprogramming fibroblasts\cite{19, 20}, called Piwil2-iCSCs. This study aimed to screen out the differentially expressed piRNAs in CSCs and further explore their effects on CSCs. PiRNA MW557525 is a novel piRNA, which, to the best of our knowledge, was discovered for the first time by our research group. We identified its differential expression in Piwil2-iCSCs. Our research shows that overexpression of piRNA MW557525 can promote the growth and the maintenance of stem cell pluripotency of Piwil2-iCSCs. All these findings indicate that novel
piRNA MW557525 may be used as an effective therapeutic target for CSCs, providing new insights into tumor diagnosis and treatment.

2. Materials and methods

2.1. Cell culture

FBs and Piwil2-iCSCs (provided by the Chongqing Key Laboratory of Child Urogenital Development and Tissue Engineering\textsuperscript{[19]}) were cultured in Dulbecco’s Modified Eagle Medium (Gibco) containing 10% fetal bovine serum (Gibco) and then incubated in an atmosphere of 5% CO2 at 37°C.

2.2. High-throughput sequencing and target gene prediction

Total RNA was isolated from Piwil2-iCSCs and FBs. Library construction and high-throughput sequencing were conducted by Shanghai Huiyan Biotechnology Co., Ltd. (Shanghai, China). Screening criteria for differentially expressed genes were P-value < 0.05 and a threshold value ≥1.5-fold change. We used SangerBox software (heatmap) to analyze the differentially expressed piRNAs. Target genes of the differentially expressed piRNAs were predicted by the miRanda algorithm (http://www.microrna.org/microrna/getDownloads.do). The targets of piRNAs were analyzed using GO (http://www.geneontology.org) and KEGG (http://www.genome.jp/dbget-bin).

2.3. Cell transfection
Piwil2-iCSCs were transfected with the PiRNA MW557525 inhibitor, PiRNA MW557525 mimics, NOP56 siRNA, and corresponding controls, which were purchased from Shanghai GeneBio (Shanghai, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for cell transfection according to the manufacturer’s protocol. Sequences of these oligonucleotides are listed in Table 1.

Table 1

PiRNA MW557525 interference and NOP56 siRNA sequences

<table>
<thead>
<tr>
<th>piRNA</th>
<th>Sequences (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW557525</td>
<td></td>
</tr>
<tr>
<td>interference</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>piRNA</td>
<td>5′-</td>
</tr>
<tr>
<td>MW557525</td>
<td>UGGAGGUGAUGAACUGUCUGAGCCUGACCUU-</td>
</tr>
<tr>
<td>Mimics</td>
<td>3′</td>
</tr>
<tr>
<td>piRNA</td>
<td>5′-UCACAACCUCCUAGAAAGAGUAGA-3′</td>
</tr>
<tr>
<td>MW557525</td>
<td></td>
</tr>
<tr>
<td>Mimics NC</td>
<td></td>
</tr>
<tr>
<td>piRNA</td>
<td>5′-</td>
</tr>
<tr>
<td>MW557525</td>
<td>AAGGUCAGGCUCAGACAGUUAUCACCUCCA-</td>
</tr>
<tr>
<td>Inhibitor</td>
<td>3′</td>
</tr>
</tbody>
</table>
2.4. RNA isolation and real-time polymerase chain reaction (qRT-PCR)

Total RNA from transfected cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNAs for miRNA and mRNA were synthesized respectively using a miRcute miRNA FirstStrand Synthesis Kit (Tiangen, Beijing, China) and Prime Script RT Master Mix (Takara, Kusatsu, Shiga, Japan) according to the manufacturers’ instructions. MiRNA quantity was measured using the miScript SYBR Green PCR Kit (Tiangen, Beijing, China), and mRNA quantity was measured using the QuantiNova™ SYBR Green PCR Kit (Qiagen GmbH, Hilden, Germany). Reactions were performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, USA). The PCR conditions were 30 s at 95°C, followed by 40 cycles at 95°C for 5 s and 60°C for 45 s. GAPDH and U6 were used as endogenous controls. The $2^{-\Delta\Delta Ct}$ method was used for analysis. The sequence-specific primers used for piRNA MW557525, U6, NOP56 and GAPDH are shown in Table 2.
Table 2

Sequence-specific primers.

<table>
<thead>
<tr>
<th>primers</th>
<th>Sequences (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>piRNA</td>
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</tr>
<tr>
<td>MW557525</td>
<td></td>
</tr>
<tr>
<td>NOP56</td>
<td>Fwd: TGAAGATCATCAACGACAATGC</td>
</tr>
<tr>
<td></td>
<td>Rev: TTCAGATAAAGACACCACACAGA</td>
</tr>
</tbody>
</table>

The internal reference primer U6 and GAPDH are brought from Sangon Biotech (Shanghai) Co., Ltd.

2.5. Western blotting

Total proteins from transfected cells were isolated using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, China) supplemented with phenylmethanesulfonyl fluoride (PMSF), and bicinchoninic acid (BCA) assay was used to detect the concentrations. The proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred the electrophoretic bands onto polyvinylidene fluoride (PVDF) membranes (MilliporeSigma, Burlington, MA, USA). Next, the membranes were blocked with 5% bovine serum albumin (ZSGB-BIO, China) for 1 h at room temperature. NOP56 mouse antibody (1:1000; Abcam, Cambridge, UK), CD24 rabbit
antibody (1:500, Santa, USA), CD133 rabbit antibody (1:1000, Abcam, UK), KLF4 rabbit antibody (1:1000, Chengdu ZEN-BIOSCIENCE, China), SOX2 mouse antibody (1:1000, Abcam, UK), BAX mouse antibody, Bcl-2 mouse antibody, and GAPDH rabbit antibody (1:1000, all three from Chengdu ZEN-BIOSCIENCE, China) were used as primary antibodies. After incubation with primary antibodies overnight at 4°C, the membranes were washed with TBST three times for at least 10 min per wash and then incubated with secondary antibody at room temperature for 1 h, followed by three washes with TBST for 10 min each. The bands were analyzed with a ChemiDoc MP Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) after treating them with an electrochemiluminescence kit (Vazyme Biotech Co., Ltd., Nanjing, China).

2.6. Cell Counting Kit-8 (CCK-8) assay

Cell proliferation assays were performed using Cell Counting kit-8 assays (MCE, China) following the manufacturer’s protocol. Piwil2-iCSCs were transfected with mimics, inhibitor, NOP56 siRNA, or NC oligonucleotides in 96-well plates with approximately 3×10^3 cells per well. Ten microliters of CCK-8 and 90 μL fresh medium were added to each well after incubating for different lengths of time (0, 1, 2, and 3 days). Optical density (OD) was measured at an absorbance wavelength of 450 nm on a microplate reader (Bio-Rad, USA) after an additional 2-h incubation.
2.7. Flow cytometry assay

After transfection, $5 \times 10^5$ cells were trypsinized and washed twice with phosphate-buffered saline (PBS). The binding buffer was used to resuspend cells, and then, $5\mu$L Annexin V-fluorescein isothiocyanate (FITC) and $5\mu$L propidium iodide (PI) were added to the buffer. The mixture was incubated for 15 min in the dark. The stained cells were detected on a flow cytometer (BD Biosciences, USA), and FlowJo software (BD Biosciences) was used to analyze the apoptosis rate.

2.8. Transwell assay

Transwell chambers precoated with Matrigel (1:8, BD, USA) were employed to check the capacity of cell invasion. After transfection, $1 \times 10^5$ cells were suspended in 200 μL of culture medium without FBS and then were seeded into the upper chamber. Meanwhile, 600-μL medium containing 10% FBS was added to the lower chamber. According to their varying migratory abilities, cells were returned to the incubator and permitted to culture for 24 h. Next, the cells in the upper chamber were carefully wiped off with water and cotton swabs. Then, 4% paraformaldehyde was used to fix the cells in the bottom chamber for 15 min. After fixing, the cells were stained with 0.5% crystal violet solution for 30 min. Images were taken using a microscope in five randomly selected fields. The migration assay was conducted identically to the
invasion assay, except that the upper chamber was not coated with Matrigel.

2.9. Dual luciferase reporter assay (LRA)

Bioinformatics prediction algorithms showed that the 3′-UTR of NOP56 contained potential binding site for piRNA MW557525. The wild-type (WT) sequence of NOP56 3′-untranslated region (UTR) harboring the binding site of piRNA MW557525 was inserted into the pGL3 vector (Shanghai GeneBio) to construct the luciferase reporter vector NOP56 3′-UTR-WT. Similarly, NOP56 3′-UTR–mutated reporter vector was established by mutating the potential target site of the piRNA. Piwi2-iCSCs were co-transfected with the recombinant-vector piRNA mimics and NC plasmid using Lipofectamine 2000. After 48 h of co-transfection, Firefly and Renilla luciferase activity were measured using a Dual LRA Kit (Promega, USA) as per manufacturer’s instructions and calculated the values based on the activity of the Renilla/Firefly luciferase genes.

2.10. Statistical analysis

GraphPad Prism 8.0.1 (GraphPad, San Diego, CA) was used to perform statistical analysis, and all data are presented as mean ± standard deviations (SDs). Two independent groups were compared by using Student’s t-test. One-way analysis of variance was used among groups. P-values lower than 0.05 were considered statistically significant. Each experiment was independently repeated at least three times.
3. Results

3.1 Analysis of the differentially expressed piRNAs and target genes

A total of 204 differentially expressed piRNAs (threshold values of \( \geq 1.5\)- and \( \leq -1.5\)-fold change and p-values < 0.05) were screened from Piwil2-iCSCs, and these piRNAs were compared with the piRNA Database (http://www.regulatoryrna.org/database/piRNA/). Of these, 159 piRNAs had been discovered and reported (45 in human genes, 114 in non-human genes), and the remaining 45 piRNAs were novel, unreported piRNAs. Of these novel, unreported piRNAs, 22 piRNAs were upregulated, while 23 piRNAs were downregulated. PiRNA MW557525 was one of the upregulated piRNAs (Fig. 1A). Then the miRanda algorithm was used to predict target genes of the differentially expressed piRNAs. A total of 77 target genes were predicted, of which \( NOP56 \) was predicted to target piRNA MW557525. GO and KEGG pathway analyses were used to analyze these target genes. GO analysis showed that these target genes mainly participate in regulating cytosolic calcium ion concentration and ATPase activity (Fig. 1B). KEGG pathway enrichment analysis showed that these target genes are mainly involved in the adenosine triphosphate (\( ATP \))–binding cassette (\( ABC \)) transporter pathway (Fig. 2C). The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; www.string-db.org) was used to carry out protein interaction network analysis (interaction score 0.15). Among the 77 target genes, myosin IXa
(MYO9A) and protein tyrosine phosphatase receptor type delta (PTPRD) each had seven interacting proteins, representing the strongest interaction points. The highest combined-score pair comprised RPS8 and RPS29 (0.999); the second-highest pair comprised RPS8 and NOP56 (0.994; Fig. 1D).

3.2. Expression of piRNA MW557525 and NOP56, and the regulate relationships of two

We identified the differential expression of piRNA MW557525 and NOP56 in Piwil2-iCSCs versus FBs. The results showed that the expression levels of piRNA MW557525 and NOP56 were significantly upregulated (Fig. 2A–B). Then the expressions of piRNA MW557525 and NOP56 after 48 h of transfection were detected to verify transfection efficiency. PiRNA MW557525 mimic transfection increased the expression of piRNA MW557525, while PiRNA MW557525 inhibitor transfection decreased piRNA MW557525 expression (Fig. 2C). In addition, siRNA NOP56 transfection decreased mRNA and protein expression levels of NOP56 (Fig. 2D–E). To confirm whether piRNA MW557525 is associated with NOP56, we examined NOP56 expression after piRNA transfection. Upregulation of piRNA MW557525 decreased mRNA and protein expression levels of NOP56, while the piRNA inhibitor increased the NOP56 expression levels (Fig. 2F–G). Furthermore, siRNA transfection increased the mRNA levels of piRNA MW557525 (Fig. 2H).
Therefore, we speculated that piRNA MW557525 might modulate *NOP56* expression. Bioinformatics prediction algorithms showed that the 3′-UTR of *NOP56* contained potential binding site for piRNA MW557525 (Fig. 2I). Then, we performed a dual LRA to confirm this prediction, and the data indicated that piRNA MW557525 did not significantly diminish the luciferase activity of *NOP56* 3′-UTR-WT versus *NOP56* 3′-UTR-MUT (Fig. 2J). Taken together, these results showed that piRNA MW557525 and *NOP56* were not directly bound but negatively correlated.

### 3.3. PiRNA MW557525 promoted proliferation, migration and invasion and inhibited apoptosis in Piwil2-iCSCs

We then performed functional experiments including CCK-8, transwell, FCM, and WB assays to detect cell proliferation, migration, invasion, and apoptosis, respectively. Results showed that the proliferative ability of Piwil2-iCSCs in the mimic transfection group was remarkably higher, and their apoptotic ability was lower than in the NC group. Downregulation of piRNA MW557525 suppressed cell proliferation and induced cell apoptosis (Fig. 3A–C). Upregulation of piRNA MW557525 significantly promoted cell migration (Fig. 3D) and invasion (Fig. 3E), while downregulation of piRNA MW557525 yielded the opposite results. Taken together, these results strongly supported the hypothesis that piRNA MW557525 might help promote the occurrence and progression of Piwil2-iCSCs.
3.4. **NOP56** inhibited proliferation, migration and invasion and promoted apoptosis in Piwil2-iCSCs

We then detected the effect of **NOP56** on Piwil2-iCSC proliferation, migration, invasion, and apoptosis. Results showed that the **NOP56** silencing group had remarkably higher proliferative ability than the NC group, and that silencing **NOP56** suppressed Piwil2-iCSC apoptosis (Fig. 4A–C). Knockdown of **NOP56** promoted Piwil2-iCSC migration (Fig. 5D) and invasion (Fig. 4E) compared with the NC group. These results suggested that **NOP56** may inhibit the occurrence and progression of Piwil2-iCSCs.

3.5. PiRNA MW557525 promoted the stem cell pluripotency of Piwil2-iCSCs, while **NOP56** suppressed the stem cell pluripotency

One of the most important characteristics of Piwil2-iCSCs was stem cell pluripotency. As **CD24**, **CD133**, **KLF4**, and **SOX2** were markers of stem cell pluripotency, we detected their expression levels via WB assays to identify the effect of piRNA MW557525 and **NOP56** on the stem cell pluripotency of Piwil2-iCSCs. Compared with the NC group, overexpression of piRNA MW557525 increased the expression of **CD24**, **CD133**, **KLF4**, **SOX2**, while downregulation of piRNA MW557525 yielded the opposite results (Fig. 5A). In addition, **NOP56** knockdown also upregulated the expression of **CD24**, **CD133**, **KLF4** and **SOX2** compared
with NC (Fig. 5B). Taken together, these results showed that PiRNA MW557525 promoted the stem cell pluripotency of Piwil2-iCSCs, while NOP56 suppressed it.

4. Discussion

Cancer is considered to be a kind of stem cell disease. In the past few decades, CSCs have been found increasingly in various malignant tumors. CSCs have many of the same characteristics as normal stem cells: for example, they remain in an undifferentiated state, express the same surface markers such as CD133 and CD44, and have relatively static or active DNA repair capabilities[5]. Unlike differentiated cancer cells, CSCs generally remain in a quiescent state or have a relatively slow cell cycle. After chemotherapy, they can be reengaged in the cell cycle, while most differentiated cells will experience death or cell cycle arrest[21]. There are currently two hypotheses on origin of CSCs: normal adult stem cells or differentiated cancer cells are reprogrammed into CSCs[5,22]. Reprogramming provides a new path for the origin of CSCs. The classical reprogramming theory posits that germline regulatory factors such as OCT4, NANOG, and SOX2 are effective factors for re-differentiating somatic cells into induced pluripotent stem cells (iPSCs)[23-25]. Studies have shown that PIWI proteins are indispensable for directly reprogramming mouse fibroblasts into pluripotent stem cells[26]. PIWI proteins are members of the Argonaute protein family and are mainly expressed in
germline stem cells or embryonic/gonadal tumor tissues of various organisms\cite{27}. PIWIL2, which is also known as a cancer/testis antigen, has been found to be highly expressed in a variety of cancers, such as oral cancer, pancreatic cancer, and papillary thyroid cancers\cite{28-30}, suggesting that it may play an important role in tumor induction, proliferation, and survival; therefore, it may serve as a prognostic predictor in cancer\cite{31}. It has also been shown that PIWIL2 may be a molecular marker for precancerous stem cells and can initiate cell reprogramming via epigenetic regulation during cervical cancer tumorigenesis\cite{32}. Transfection of mouse embryonic fibroblasts with PIWIL2 gene can produce cancer stem cell-like cell lines\cite{33}.

Our research group used the PIWIL2 gene to reprogram human foreskin fibroblasts in the early stage and induced cells with the same characteristics of CSCs in vitro, which were called Piwil2-iCSCs\cite{19}. The high expression of the PIWIL2 gene may be the key to distinguishing Piwil2-iCSCs from normal somatic cells. As a small RNA that interacts with PIWI proteins, piRNAs may play an important role in the reprogramming and successful construction of Piwil2-iCSCs. Of late, it has been proven that piRNA-823 is highly expressed in breast cancer, especially in CSCs, and the difference is very significant\cite{34}. Whether and how piRNAs regulate human CSCs remain unclear. On this basis, we performed high-throughput sequencing on the constructed cell line Piwil2-iCSCs in order to screen out
differentially expressed piRNAs and further explain the regulatory effect of piRNAs on CSCs.

In this study, the sequencing results showed that a total of 204 differentially expressed piRNAs in piwil2-iCSCs were screened, of which 123 piRNAs were significantly upregulated and 81 piRNAs were significantly downregulated. We compared them with the piRNA database and the current existing literature, and found that 45 piRNAs have not been previously reported. piRNA MW557525 is one of the highly expressed piRNAs. We further verified its expression in Piwil2-iCSCs, and the results showed that the expression level of piRNA MW557525 is consistent with the sequencing results. We performed target gene prediction for the differentially expressed piRNAs, and a total of 77 target genes were predicted. The predicted target gene of PiRNA MW557525 is NOP56, which is a component of the small molecule nucleolar ribonucleoprotein complex that can participate in the modification of ribosomal RNA precursor ribose methylation[35]. Studies have shown that NOP56 is highly expressed in many cancers, such as acute lymphoblastic leukemia, colon cancer, and Burkitt lymphoma, and it may be related to cancer metastasis and recurrence[36-38]. However, there are also studies showing that the high expression of NOP56 can improve the prognosis of metastatic clear cell renal cell carcinoma[39]. Since the expression and role of NOP56 in cancers have not yet reached a unified conclusion, its expression and regulation in
CSCs deserve more attention in our study. GO and KEGG enrichment analyses of the predicted target genes showed that these target genes are mainly involved in the regulation of calcium ion concentration and ATPase activity. Studies have shown that there is abnormal energy metabolism in cancers and CSCs. Since mitochondria are significantly fewer in cancers and CSCs than in normal cells, CSCs mainly produce energy through glycolysis pathway\cite{40,41}. We speculate that these piRNAs may be involved in regulating the energy metabolism of CSCs. However, since the P-value of GO analysis is higher than 0.05, this conclusion is only for reference.

Cell functional experiments showed that piRNA MW557525 promoted the proliferation, migration, and invasion of Piwil2-iCSCs and inhibited their apoptosis, while \textit{NOP56} had the opposite effect. However, \textit{NOP56} was also confirmed to be highly expressed in Piwil2-iCSCs. We speculate that increasing the expression level of \textit{PIWIL2} in fibroblasts will not only change the expression of piRNA MW557525, but will also regulate the expression of other genes that can target \textit{NOP56}, thus increasing the overall expression of \textit{NOP56} in Piwil2-iCSCs. We predicted the binding site of piRNA MW557525 and \textit{NOP56} through bioinformatics algorithms, but the result of dual luciferase was negative, indicating that piRNA MW557525 and \textit{NOP56} do not bind directly. We further verified the interaction between \textit{NOP56} and piRNA MW557525, and the results demonstrated that there is a negative correlation. PiRNAs are different
from miRNAs. Most piRNAs are not complementary to the mRNAs of potential target genes. This indicates that piRNAs may be involved in epigenetic regulation rather than post-transcriptional regulation to control a variety of biological phenomena including cancers\[^42\]. Therefore, we speculate that piRNA MW557525 may indirectly regulate the expression of \textit{NOP56}.

Current research holds that genes such as \textit{SOX2} and \textit{KLF4} are involved in somatic cell reprogramming into CSCs\[^43, 44\]. \textit{CD24} and \textit{CD133} have been confirmed as markers of CSCs for a variety of tumors\[^7, 9, 10\]. The high expression of \textit{PIWIL2} gene leads to the differential expression of piRNAs. We guess these differential piRNAs are involved in regulating the expression of the above genes, leading to the reprogramming of fibroblasts into cells similar to CSCs. Therefore, we further tested the effects of piRNA MW557525 and \textit{NOP56} on the expression levels of \textit{CD133}, \textit{CD24}, \textit{SOX2}, and \textit{KLF4}. The results showed that piRNA MW557525 upregulated the expression of the above genes, while \textit{NOP56} downregulated the expression of the above genes. Therefore, we speculate that piRNA MW557525 regulates the expression of \textit{SOX2} and \textit{KLF4} to participate in the process of reprogramming somatic cells into CSCs, and that it regulates the expression of \textit{CD133} and \textit{CD24} to enable Piwil2-CSCs to maintain their stem cell pluripotency and maintain their undifferentiated state.
In this study, we screened the differentially expressed piRNAs in Piwil2-iCSCs, and predicted target genes. We identified that piRNA MW557525 was negatively correlated with NOP56, and it promoted the proliferation, migration, invasion, and inhibited apoptosis in Piwil2-iCSCs, and it also promoted the expressions of CD24, CD133, KLF4, and SOX2, while NOP56 showed the opposite effect.

5. Conclusions

In summary, our research demonstrated that piRNA MW557525 is a novel piRNA selected by HTS. To the best of our knowledge, our research group is the first to study and report this piRNA. We speculate that it may be involved in the process of reprogramming somatic cells into CSCs and can regulate the growth of CSCs, while its target gene NOP56 showed the opposite effect. We also identified that piRNA MW557525 was negatively correlated with NOP56. These findings provided a new direction for exploring the role of piRNAs in CSCs. Since piRNAMW557525 is a novel piRNA, there are no other studies that detail in-depth research on it. It is arduous to study it in depth. We will continue to look for its direct target gene and explore the relevant mechanisms involved in the regulation of CSC reprogramming.

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**Conflicts of interest:** The authors declare that they have no competing interests.

**[Authors' contributions]**

Conceptualization: [Liming Jin, Zhaoxia Zhang and Dawei He]; Data curation: [Liming Jin, Zhaoxia Zhang, Lianju Shen and Chunlan Long]; Formal analysis: [Liming Jin and Zhaoxia Zhang]; Funding acquisition: [Guanghui Wei and Dawei He]; Methodology: [Zhang Wang, Liming Jin and Zhaoying Wang]; Writing – original draft: [Zhaoxia Zhang and Liming Jin]; Resources: [Dawei He]; Supervision: [Dawei He and Guanghui Wei].
Reference:


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Figure legend

**Figure 1. Analysis of differentially expressed piRNAs and target genes**
A: Heatmap of differentially expressed piRNAs in Piwil2-iCSCs. The difference between duplicate groups may be due to measurement errors. B-C: GO and KEGG analyses of the target genes. Regulation of cytosolic calcium ion concentration, ATPase activity, and ABC transporters pathway were the most possible processes involved in these target genes. The results of GO analysis showed that the P-value was greater than 0.05, so the results were for reference only. D: Protein interaction analysis of the target genes. Higher combined scores indicated greater credibility, and proteins with more interaction points were more important in PPI. The highest combined score pairs were RPS8 and RPS29. PTPRD and MYO9A had the most interaction points.

**Figure 2. Expression of piRNA MW557525/NOP56 and the regulate relationships of two**
A–B: Expression of piRNA MW557525 and NOP56 in Piwil2-iCSCs compared with FBs as shown by qRT-PCR. C: PiRNA MW557525 expression was measured via qRT-PCR to confirm transfection efficiency. D–E: NOP56 mRNA and protein expression levels were assayed respectively via qRT-PCR and WB after transfection. F–G: Effect of piRNA MW557525 transfection on NOP56 expression. H: Effect of NOP56 silencing on piRNA MW557525 expression. I: Binding sequences between piRNA MW557525 and NOP56. J: LRA of cells after
co-transfection with WT or MUT NOP56 3′-UTR and piRNA MW557525. For comparisons, Student’s t-test was performed (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 3. PiRNA MW557525 promoted cell proliferation, migration and invasion and inhibited apoptosis Piwil2-iCSCs were transfected with piRNA MW557525 mimics, inhibitor or NC. **A:** We performed a CCK-8 assay to determine cell proliferation. **B–C:** Cell apoptosis was detected by FCM and WB assays. **D–E:** Transwell assays were performed to assess migration and invasion ability of Piwil2-iCSCs. For comparisons, Student’s t-test was performed (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 4. NOP56 knockdown promoted cell proliferation, migration and invasion and suppressed apoptosis Piwil2-iCSCs were transfected with NOP56 siRNA or NC siRNA. **A:** Cell proliferation was assessed at the indicated time points via CCK-8 assay. **B–C:** FCM and WB analyses were performed to determine cell apoptosis. **D–E:** Transwell assays were performed to evaluate migration and invasion ability (*, $P < 0.05$; **, $P < 0.01$).

Figure 5. PiRNA MW557525 and NOP56 regulated the stem cell pluripotency of Piwil2-iCSCs **A:** CD24, CD133, KLF4, SOX2, and GAPDH expression levels were detected via WB after 72 h of transfection with piRNA MW557525 mimics, inhibitor, or NC. **B:** Relative expression
of CD24, CD133, KLF4, SOX2, and GAPDH were detected via WB after 72 h of transfection with NOP56-specific siRNAs (*, P < 0.05).
Figure 4

A. Absorbance at 450nm over time for NC siRNA and NOP56 siRNA.

B. Flow cytometry analysis for apoptosis rate for NC siRNA and NOP56 siRNA.

C. Western blot analysis for BAX, BCL2, and GAPDH with molecular weights of 21 kDa, 26 kDa, and 36 kDa, respectively.

D. Images showing NC siRNA and NOP56 siRNA with migration cell numbers.

E. Images showing NC siRNA and NOP56 siRNA with invasion cell numbers.
Figure 1

Analysis of differentially expressed piRNAs and target genes. A: Heatmap of differentially expressed piRNAs in Piwil2-iCSCs. The difference between duplicate groups may be due to measurement errors. B-C: GO and KEGG analyses of the target genes. Regulation of cytosolic calcium ion concentration, ATPase.
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Figure 2
Expression of piRNA MW557525/NOP56 and the regulate relationships of two A–B: Expression of piRNA MW557525 and NOP56 in Piwi2-iSCs compared with FBs as shown by qRT-PCR. C: PiRNA MW557525 expression was measured via qRT-PCR to confirm transfection efficiency. D-E: NOP56 mRNA and protein expression levels were assayed respectively via qRT-PCR and WB after transfection F–G: Effect of piRNA MW557525 transfection on NOP56 expression. H: Effect of NOP56 silencing on piRNA MW557525 expression. I: Binding sequences between piRNA MW557525 and NOP56. J: LRA of cells after co-transfection with WT or MUT NOP56 3′-UTR and piRNA MW557525. For comparisons, Student’s t-test was performed (*, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \)).
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**PiRNA MW557525 promoted cell proliferation, migration and invasion and inhibited apoptosis** Piwil2-iCSCs were transfected with piRNA MW557525 mimics, inhibitor or NC. **A:** We performed a CCK-8 assay to determine cell proliferation.  
**B–C:** Cell apoptosis was detected by FCM and WB assays. **D–E:** Transwell assays were performed to assess migration and invasion ability of Piwil2-iCSCs. For comparisons, Student’s t-test was performed (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001).
**Figure 4**

**NOP56** knockdown promoted cell proliferation, migration and invasion and suppressed apoptosis. Piwil2-iCSCs were transfected with **NOP56** siRNA or NC siRNA. A: Cell proliferation was assessed at the indicated time points via CCK-8 assay. B–C: FCM and WB analyses were performed to determine cell apoptosis. D–E: Transwell assays were performed to evaluate migration and invasion ability (*, $P < 0.05$; **, $P < 0.01$).
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PiRNA MW557525 and NOP56 regulated the stem cell pluripotency of Piwil2-iCSCs A: CD24, CD133, KLF4, SOX2, and GAPDH expression levels were detected via WB after 72 h of transfection with piRNA MW557525 mimics, inhibitor, or NC. B: Relative expression of CD24, CD133, KLF4, SOX2, and GAPDH were detected via WB after 72 h of transfection with NOP56-specific siRNAs (*, P < 0.05).