



circ0093740 Promotes Tumor Growth and Metastasis by Sponging miR-136/145 and Upregulating DNMT3A in Wilms Tumor

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As a research hotspot, circular RNAs (circRNAs) is one type of non-coding RNAs which have many different functions in biological processes. However, there is lack of study investigating the underlying molecular mechanism and the potential roles of circRNAs in Wilms tumor. We conducted a high-throughput microarray sequencing to screen differentially expressed circRNAs in Wilms tumor. A novel circRNA (circ0093740) was identified as a frequently upregulated circRNA in Wilms tumor cells and tissues. Suppression of circ0093740 remarkably inhibited the proliferation and migration ability in Wilms tumor, validated by several experiments. The molecular mechanism of circ0093740 was investigated by luciferase assays and RNA immunoprecipitation assays. The results revealed that circ0093740 promotes the growth and migration ability by sponging miR-136/145 and upregulating DNMT3A. In conclusion, our study discovered the biological role of the circ0093740-miR-136/145-DNMT3A axis in Wilms tumor growth and metastasis which is important for developing new treatment strategy.

Keywords: circ0093740, DNMT3A, Wilms tumor, circular RNAs, competitive endogenous RNAs

INTRODUCTION

Nephroblastoma (also known as Wilms tumor) is a kind of mixed embryonal tumor, which accounts for over 7% of all childhood malignancy and 90% of childhood renal tumors (1). The combination of nephrectomy surgery and chemotherapy was the most common and effective treatment of Wilms tumor. More than 90% of patients with Wilms tumor can be completely cured by systematic therapy (2). However, a part of children with high-risk histology Wilms tumor do not respond to conventional therapy and relapse a few years after surgery (3). Therefore, it is important for researchers to seek for the molecular pathogenesis of Wilms tumor and develop novel treatment strategies to improve outcomes in patients with high-risk Wilms tumors.

CircRNAs are one novel type of single strand non-coding RNAs transcripts in cells, which play very important role in regulating genes *via* different molecular mechanism (4). circRNAs are highly represented in the eukaryotic transcriptome which are originated from the back-spliced sequences of exonic or intronic pre-mRNAs (precursor mRNAs) sequences without an upstream head or a downstream tail (5). With a circular structure, they are very stable and resistant to RNA exonuclease which are abundant in most mammalian tissues compared to the linear mRNA transcription (6). In the intracellular microenvironment, circRNAs can regulate the expression of vital oncogenes *via* multiple comprehensive molecular mechanisms, including interacting with proteins, binding microRNAs, and encoding new small molecular proteins (7). Thanks to RNA-seq technology and bioinformatic analysis, lots of circRNAs has been identified (8). circRNAs have been discovered as regulators of a diversity of diseases, including heart failure, neurological disorders, diabetes, and cancers (9). Taking the most famous *cIRS-7/cdr1as* as an example, this circRNA promotes the growth, migration, chemotherapy resistance, and immune deficiency by sponging miR-7 in multiple types of tumors (10–14). Acts as a tumor suppressor, circFBXW7 is low expressed in tumor tissues which can suppress growth in glioma and breast cancer by sponging miRNA and translating a small 21kda FBXW7 new protein (15, 16). Regulated by TNRC6A, circ0006916 was discovered as tumor promoter in lung cancer cells (17). In addition, circPLK1 were proven as a tumor promoting circRNA by reducing apoptosis in breast cancer (18, 19). The self-renewal and the stemness of colon tumor-initiating cells is enhanced by circCTIC1 by upregulating BPTF-dependent c-Myc expression in colon cancer (20). However, there is lack of study investigating the underlying molecular mechanism and the potential roles of circRNAs in Wilms tumor.

We firstly conducted a high-throughput microarray to screen for the differentially expressed circRNAs in Wilms tumor. We identified a novel circRNA (circ0093740) as a frequently upregulated circRNA in Wilms tumor cells and tissues. Suppression of circ0093740 remarkably inhibited the proliferation and migration ability in Wilms tumor, validated by several experiments. The molecular mechanism of circ0093740 was investigated by luciferase assays and RIP assays. Generally, we discovered the biological functions of the circ0093740-miR-136/145-DNMT3A axis in Wilms tumor growth and metastasis which is important for developing new treatment strategy.

MATERIALS AND METHODS

Clinical Sample Data

Fresh primary Wilms tumor samples and adjacent normal kidney samples were collected from Sun Yat-sen University Cancer Center (SYSUCC) and were frozen in liquid nitrogen at once. This study was approved by the Ethics Committee of the SYSUCC and performed in accordance with the Declaration of Helsinki.

Cell Culture

Cell lines used in this study including SKNEP1, G401, HANB, and HEK293T were cultured in DMEM (Gibco, USA) containing 10% FBS (Gibco). All cell lines was verified by DNA fingerprinting.

Western Blot Analysis

The protein was extracted by RIPA and added with PMSF to prevent degradation. The protein was transferred to the PVDF membranes for 2 h at 300 mA and incubated at 4°C with primary antibody (1:1000) overnight and then exposed with the secondary antibody at room temperature for 1 hour. Primary antibody anti-DNMT3A (1:1000, Abcam, USA) and anti-TP53 antibody (1:1000, CST, USA) are used to detect certain protein.

RT-qPCR Analysis

TRIzo (Invitrogen, USA) was utilized to extract cellular RNA. qRT-PCR assays were conducted with SYBR Premix Ex Taq Kit (Takara, Japan). The primers for circ0093740 are F: 5'-GTGATTGCCGTCCACTCACT-3'; R: 5'-AGTTCTATGTGGGGTCTGGT-3'. The primers for PCDH15 are F: 5'-AGCACCGGAAGAGTTCTGGAT-3'; R: 5'-ACCACTATTCGCACTTCATGGTA-3'. The primers for ACTB are F: 5'-CATGTACGTTGCTATCCAGGC-3'; R: 5'-CTCCTTAA TGTCACGCACGAT-3'.

Actinomycin D Assay

SKNEP1 Wilms tumor cells were exposed to 5 μ g/ml actinomycin D (MCE) to degrade the linear mRNA transcription at 0-, 8-, 16-, and 24-hour time point. Linear PDCH15 mRNA and circRNA circ0093740 were tested by RT-qPCR analysis.

RNase R Digestion Assay

After 1 μ g extracted total RNA of SKNEP1 Wilms tumor cell line was incubated with the RNase R (5 U/ μ g) or ddH₂O for 30 minutes at RT, the remaining RNA solution was purified and quantified by RT-qPCR analysis.

CCK-8 Assay

SKNEP1 and G401 Wilms tumor cells were digested and then resuspended, and si-circ0093740 (3000 cells per well) and si-control cancer cells (3000 cells per well) were seeded into a 96-well plate. The cells were incubated for two days at 37°C. Afterwards, CCK-8 solution (10 μ l) was added to each well of the 96-plate before incubating for two hours.

Transwell Assay

Overall, 5×10^4 tumor cells were resuspended and added to the upper chambers (without FBS) and medium (medium containing 20% FBS) was added to the lower chambers. After fix and staining with crystal violet (1.5%), the migrated cells were imaged.

Luciferase Reporter Assay

SKNEP1 and G401 Wilms tumor cells were seeded into 5×10^3 cells in each well (96-well plate). The predicted miR-136 and miR-145 binding sites of circ0093740, and 3'-UTR of DNMT3A was mutated. Afterwards, the miRNA inhibitors or mimics and constructed reporting vectors (circ0093740-wt/mut or DNMT3A 3'-UTR-wt/mut) were cotransfected into cells for 48 hours before further examination.

RNA Immunoprecipitation (RIP)

The RIP assays for the AGO2 protein were performed with an anti-AGO2 antibody. The relative expression level of circ0093740,

DNMT3A, and miR-136/145 was tested after RNA purification. SKNEP1 and G401 Wilms tumor cells were immediately transfected with MS2bs-circ0093740 vector, MS2bs-circ0093740-mt vector and MS2bs-Rluc vector. The abundance of miR-136/145 was determined after the purification of RNA complexes.

Mouse Xenograft Assay

SKNEP1 Wilms tumor cells (2×10^7) were subcutaneously injected into nude mice (four mice for each group, 4-week-old) and treated with intratumoral injection (50 μ L si-circ0093740, or si-control) every four days. After 28 days, mice were euthanized. The volume of tumors was estimated every four days according to the following formula: $0.5 \times \text{width}^2 \times \text{length}$. For mouse lung metastasis assay, SKNEP1 cells (3×10^5) were injected through tail veins of nude mice (four mice for each group). The lungs were excised after 8 weeks and the number of metastatic sites were quantified.

Statistical Analysis

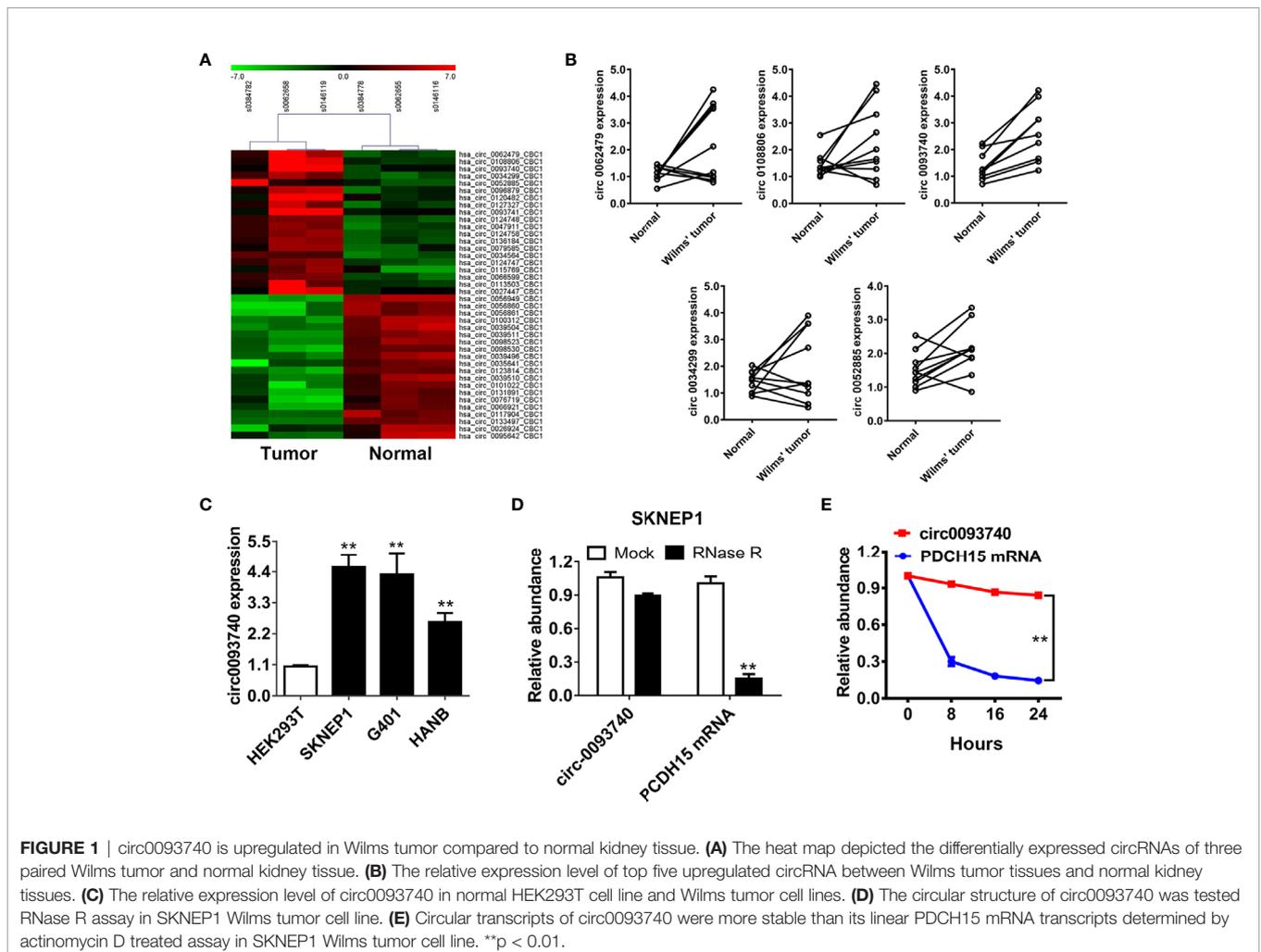
All statistical analysis was performed with SPSS 23.0 software (SPSS, USA). Groups were compared using Student's t test. Paired t test was used to compare the expression of circ0093740 in two matched

groups. All data are reported as the mean \pm standard deviation (SD). $P < 0.05$ was considered as statistically significant.

RESULTS

circ0093740 Is Upregulated in Wilms Tumor Compared to Normal Kidney Tissue

To depict the expression profile of circRNAs in Wilms tumor, a circRNAs microarray assay was conducted using three pairs of Wilms tumor patient tissues and adjacent normal kidney samples (Figure 1A). Afterwards, we further examined the expression level of the top five upregulated circRNAs in ten pairs of Wilms tumor patient tissues and normal kidney tissues (Figure 1B). Among them, circ0093740 was significantly upregulated in all tumor-normal pairs. circ0093740 was overexpressed in Wilms tumor lines compared to normal kidney cell HEK293T, especially in SKNEP1 and G401 cell lines (Figure 1C). We found that the expression of circ0093740 was higher in SKNEP1 and G401, rather than HANB. Therefore, we chose SKNEP1 and G401 for further examination in this study. The actinomycin D assays and the RNase R assays were then carried out



to confirm the circular structure and stability of circ0093740. The results revealed that circ0093740 was resistant to RNA exonuclease (Figure 1D). circ0093740 has longer half-life span than the linear PDCH15 mRNA in SKNEP1 cell line (Figure 1E).

Suppression of circ0093740 Inhibits the Proliferation of Wilms Tumor Cells

We conducted functional assays to explore the potential role of circ0093740 in Wilms tumor progression. circ0093740 was reduced after transfected with siRNAs (sequence anti-back-splicing junction region of circ0093740) which was verified in SKNEP1 and G401 Wilms tumor cell lines (Figure 2A). Inhibition of circ0093740 suppressed proliferation ability of SKNEP1 and G401 cell lines *in vitro*, revealed by CCK-8 assays (Figure 2B). Further validation of the function of circ0093740 in mouse xenograft assays was conducted. Tumor volumes curves showed that inhibition of circ0093740 could remarkably suppress tumor growth at each time point (Figure 2C). Moreover, we found that the Ki67 expression was remarkably decreased in the circ0093740 knockdown group in tumor tissues (Figure 2D).

Suppression of circ0093740 Inhibits the Metastasis of Wilms Tumor Cells

We next performed migration and invasion associated assays. Downregulation of the expression of circ0093740 could significantly inhibit the percentage of wound healing ability of SKNEP1 and G401 Wilms tumor cells (Figure 3A). Transwell assay showed that silence of circ0093740 could reduce the migration ability of SKNEP1 and G401 cells (Figure 3B). In consistent with the results of *in vitro* experiments, suppression of

circ0093740 could also inhibit the metastatic ability of SKNEP1 and G401 Wilms tumor cells in lung metastasis experiment *in vivo* (Figure 3C).

circ0093740 Serves as a Sponge of miR-136/145 in Wilms Tumor

circ0093740 was predominantly expressed in the cytoplasm of cell, detected by qPCR analysis of different cell fraction (Figure 4A). As a result, we conducted a miRNA sequencing on three pairs of circ0093740 overexpression and vector samples in SKNEP1 cell line (Figure 4B). Circular RNA Interactome database was employed. miR-136 and miR-145 were predicted to interact with circ0093740 and downregulated in circ0093740 overexpression group (Figure 4C). In Wilms tumor cell lines, miR-136 and miR-145 were both downregulated detected by RT-qPCR analysis (Figure 4D). The results showed that the luciferase activity was significantly reduced after the co-transfection of wild type reporter vectors and miR-136/145 mimics (Figure 4E). Ago2-related RIP assays were further conducted to confirm the direct interaction between circ0093740 and miR-136/145. Both miR-136 and miR-145 were predominantly gathered in the MS2bs-circ0093740 vector overexpressed group (Figure 4F).

circ0093740 Facilitates Wilms Tumor Progression Through circ0093740-miR-136/145-DNMT3A Axis

Then, TargetScan algorithm was utilized to predict the downstream targeted oncogenes of miR-136 and miR-145. Among the genes, DNMT3A were identified as putative

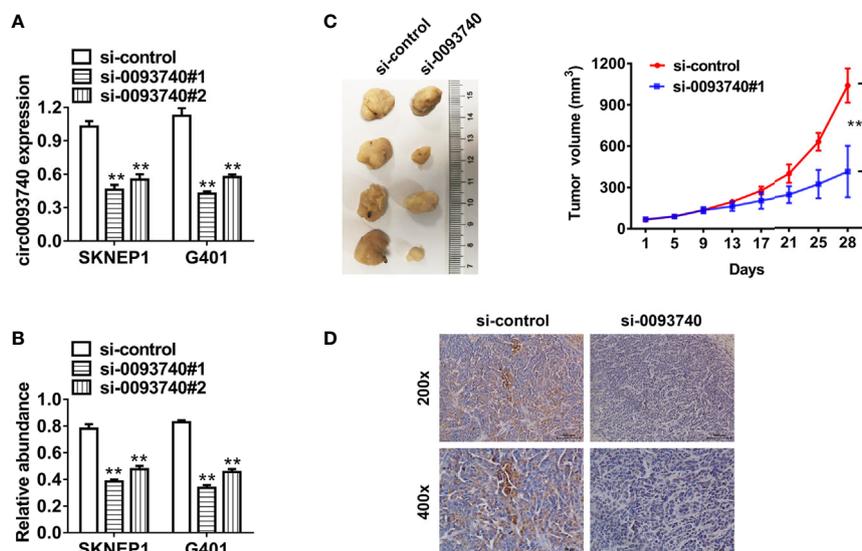


FIGURE 2 | Suppression of circ0093740 inhibits the proliferation of Wilms tumor cells. **(A)** siRNA knockdown of circ0093740 was validated in SKNEP1 and G401 Wilms tumor cell line. **(B)** CCK-8 assays were conducted to evaluate cell proliferation in SKNEP1 Wilms tumor cell line. **(C)** Mouse xenograft models of SKNEP1 Wilms tumor cell line was established. Tumor volume was estimated in every four days. **(D)** The images of Ki-67 IHC expression are presented. ***p* < 0.01.

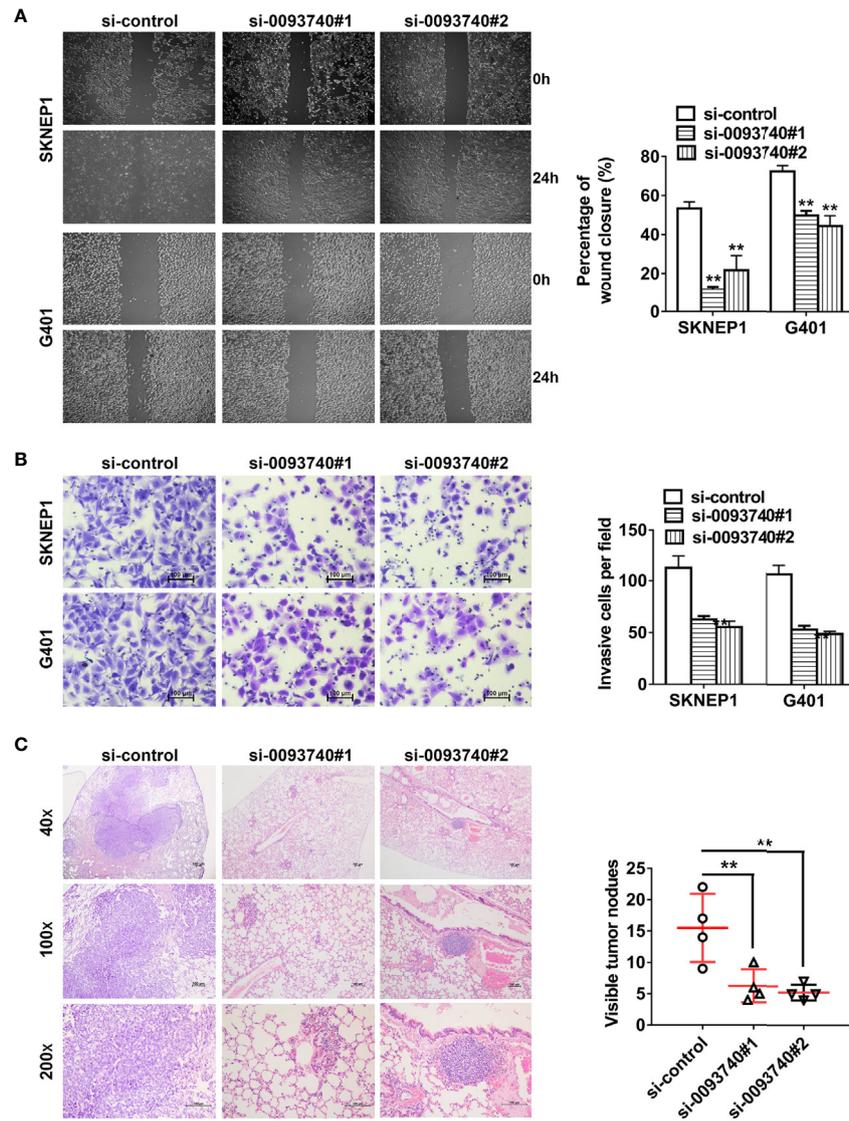


FIGURE 3 | Suppression of circ0093740 inhibits the metastasis of Wilms tumor cells. **(A)** Wound healing assays were conducted in SKNEP1 and G401 Wilms tumor cell line. **(B)** Transwell assay was performed to investigate the migration ability of SKNEP1 and G401 Wilms tumor cell line. **(C)** The number of lung metastases was counted and recorded. HE-stained tumor sections of lung metastases were presented. ** $p < 0.01$.

downstream target oncogene of both miR-136 and miR-145 (**Figure 5A**). We conducted qPCR analysis and found that DNMT3A was remarkably upregulated in Wilms tumor (**Figure 5B**). Overexpression of miR-136 and miR-145 contributed to the reduction of DNMT3A mRNA expression level (**Figure 5C**). Luciferase activity was extremely decreased after cotransfection of miR-136/145 mimics and 3'-UTR-DNMT3A reporters in SKNEP1 and G401 Wilms tumor cell lines. After transfection of the mutated reporting vector (mutation of predicted binding site), relative luciferase activity remained unchanged (**Figure 5D**). In addition, we conducted AGO2 related RIP assays which revealed circ0093740, miR-136/145 and DNMT3A were all enriched to AGO2 RNA binding

protein in both SKNEP1 and G401 Wilms tumor cell lines (**Figure 5E**). Enrichment to RNA induced silencing complex (RISC) of DNMT3A was remarkably increased after knockdown of circ0093740 (**Figure 5F**). Inhibition of circ0093740 could incredibly decrease the protein level of DNMT3A and increase the protein level of tumor suppressor TP53, analyzing by western blot assays (**Figure 5G**).

DISCUSSION

With covalently closed loop, circRNAs are widely expressed in mammal tissues with tissue-specific patterns (21). Trans-acting

Animal Care and Use Committee of Sun Yat-sen University Cancer Center.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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