Running title: miR-200c-3p suppresses nephroblastoma cells

microRNA-200c-3p suppresses proliferation and invasion of nephroblastoma cells by targeting EP300 and inactivating the AKT/FOXO1/p27 pathway

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miR-200c-3p is aberrantly expressed in numerous cancers, but its underlying mechanisms in nephroblastoma are unknown. In our study, the differentially regulated miRNAs between the nephroblastoma tissues and adjacent non-neoplastic renal tissues were screened based on microarray analysis. The miR-200c-3p expression in nephroblastoma tissues and cells was detected by qRT-PCR. Then, the effects of miR-200c-3p mimic or inhibitor on cell proliferation, invasion, and migration were evaluated by CCK-8 assay, plate colony formation assay, soft agar assay, Transwell, and wound-healing assay in SK-NEP-1 and G401 cells. Afterward, the target gene of miR-200c-3p was predicted by TarBase, miRTarBase, miRDB softwares, and then verified by dual-luciferase reporter gene assay. The in vivo effects of miR-200c-3p on pathological changes and tumor volume were investigated in tumor xenograft mice by H&E staining and in vivo fluorescence imaging. ChIP assay was used to evaluate the relationship between histone acetyltransferase E1A-binding protein p300 (EP300) and P27, and the relationship of the role of miR-200c-3p in nephroblastoma and the AKT/FOXO1/p27 signaling pathways was evaluated by western blotting. Our study shows that miR-200c-3p was downregulated in nephroblastoma tissues and cells, and EP300 was a target gene of miR-200c-3p. Furthermore, miR-200c-3p mimic decreased cell proliferation and inhibited cell migration and invasion in nephroblastoma. Mechanistically, miR-200c-3p could inhibit p-AKT activity and enhance p-FOXO1 and p27 expression. Notably, the transcription factor P27 could bind to the EP300 promoter. This study demonstrates a new approach to treat nephroblastoma.

Key words: miR-200c-3p; nephroblastoma; EP300; AKT/FOXO1 pathway; proliferation; invasion

Nephroblastoma is an embryonal kidney neoplasia and is found in children younger than five years old. Unfortunately, the relapse rate of nephroblastoma is up to 15% [1-3]. Therefore, to investigate the mechanism of nephroblastoma development and pathogenesis is very important. These reports showed aberration expression of WT1, abnormalities of 11p15 methylation, and Wnt-activating mutations in nephroblastoma were usually common. In recent years, the comprehensive genomic analyses of nephroblastoma identified novel mutations in DROSHA, DGCR8, and DICER1 genes due to the aberrantly regulation of miRNA [4-6]. Despite these efforts, recurrent driver mutations were not identified in most nephroblastomas [7].

microRNAs (miRNAs), is transcribed by RNA polymerase II to produce small noncoding RNA(19-25 nt) [8, 9]. These reports showed that miRNAs can bind to the complementary sequences of the 3'-untranslated region (3'-UTR) in their target mRNAs to inhibit about 20-30% gene mRNA transcription and protein translation [10, 11]. In multiple cancers, miRNAs can bind 3'-UTR of oncogenes or tumor-suppressor genes to downregulate the gene expression and participate in the cancer metastasis [12]. Although miRNAs played key roles in cancer progression, the detailed molecular regulatory mechanisms of miRNAs in cancer were not well understood. In our research, we investigated the expression of miR-200c-3p in nephroblastoma and further explored its target gene and molecular mechanisms, which might provide novel targets for the treatment of nephroblastoma.

In this study, our study showed that miR-200c-3p was downregulated in nephroblastoma tissues and cells, which played a key role in the progression of nephroblastoma. Moreover, we found that miR-200c-3p inhibited the proliferation and invasion of nephroblastoma by targeting EP300 and inactivating AKT/FOXO1/p27 pathway. The molecular mechanisms might provide new insights and therapeutic strategies for nephroblastoma prevention and treatment.

Patients and methods

Cell lines and human tissue samples. Human nephroblastoma cell lines SK-NEP-1, G401 and human embryonal kidney cells 293FT cells were obtained from Shanghai Cell Bank of Type Culture Collection (Shanghai, China). SK-NEP-1 cells were cultured in McCoy's 5A medium (HyClone, Logan, Utah, USA) and G401 cells were cultured in RPMI-1640 medium (HyClone, Logan, Utah, USA). All cell lines supplemented with 10% fetal bovine serum (HyClone, Logan,

Utah, USA) at 37 °C under 5% CO₂. Fresh nephroblastoma tissues and adjacent non-neoplastic renal tissues were collected from 22 patients who underwent nephroblastoma resection without prior radiotherapy and chemotherapy at the Department of Urology Surgery in Shenzhen Children's Hospital (Shenzhen, Guangdong Province, People's Republic of China) in 2018. These samples were collected immediately after resection, snap-frozen in liquid nitrogen, and stored at -80 °C until needed.

The study protocol was performed in accordance with the guidelines outlined in the Declaration of Helsinki. For the clinical patient tissues used in this study, prior patient consent and approval from the Institutional Research Ethics Committee (IREC) were obtained.

Microarray analysis. The miRNA expression patterns among adjacent non-neoplastic renal tissues (Normal) and nephroblastoma tissues without lymphatic metastasis were analyzed using human miRNA Paraflo™ array (CapitalBio Technology laboratory, Beijing, China). Three clinical samples were pooled in each group.

Construction of plasmids and transfection. Lentiviral constructs expressing miR-200c-3p (Lenti-miR microRNA precursor clone collection; System Biosciences, Carlsbad, CA, USA) were packaged using the pLP1/pLP2/pLP VSV-G lentivector Packaging Kit (Invitrogen, Foster city, CA, USA). Lentiviral constructs were used to infect nephroblastoma cell lines to establish cells stably expressing miR-200c-3p. miR-200c-3p inhibitor and its negative control antisense oligos, were obtained from Genechem Company (Shanghai, China), and were used to transfect indicated cells, according to the manufacturer.

Cell proliferation assay. For analysis of the impacts of miR-200c-3p on cell proliferation in nephroblastoma, 1×10^3 SK-NEP-1 and G401 cells were respectively seeded into 96-well plates, and then transfected with miR-200c-3p mimic, mimic NC, miR-200c-3p inhibitor or NC inhibitor using the Lipofectamine™ 3000 transfection reagent (Thermo Fisher Scientific, USA). At each time point, 10 µl CCK-8 assay reagent (Beyotime, China) was added into each well of the 96-well plates. The absorbance density at a wavelength of 450 nm was measured after 2 h of incubation at 37 °C. Each cell group was plated in three duplicate wells. All experiments were performed three times.

Plate colony formation assay. Totally, 1×10^2 G401 cells were added to each well of 6-well culture plates, and then transfected with miR-200c-3p mimic, mimic NC, miR-200c-3p inhibitor or NC inhibitor using the Lipofectamine™ 3000 transfection reagent (Thermo Fisher Scientific, USA).

106 The cells were incubated at 37 °C for 14 days. Then the colonies were washed twice with
107 phosphate-buffered saline, fixed with 4% paraformaldehyde for 5 min and stained with Giemsa
108 solution. The number of colonies containing 50 cells or more was counted under a microscope
109 [plate clone formation efficiency=(number of colonies/number of cells inoculated)] × 100%).

110 **Cell cycle assay.** Briefly, the SK-NEP-1 and G401 cells that transfected with miR-200c-3p mimic,
111 mimic NC, miR-200c-3p inhibitor or NC inhibitor were harvested by trypsin and fixed by 70%
112 ethanol. Following cell centrifugation, the cells were stained with 10 µg/ml of propidium iodide (PI)
113 for 30 min at room temperature. Lastly, the cell cycle of the treated cells were observed and
114 analyzed using a FACScan flow cytometer (BD Biosciences , USA) thereafter. The proliferative
115 index was calculated as $(S+G2/M)/(G0/G1+S+G2/M) \times 100\%$.

116 **Transwell matrigel invasion assay.** Briefly, 8 µm Boyden invasion chambers were rehydrated
117 with RPMI 1640 (serum-free) for 2 h at 37 °C. Next, 1.5×10^5 G401 cells in serum-free RPMI 1640
118 were seeded to the upper compartment of the chamber cells, and treated with miR-200c-3p mimic,
119 mimic NC, miR-200c-3p inhibitor or NC inhibitor. Then RPMI 1640 culture medium containing
120 20% fetal bovine serum was added to the lower compartment as the chemotactic factor and the
121 chambers were incubated at 37 °C for 24 h. The noninvasive cells were removed with a cotton swab.
122 Cells that migrated through the membrane and stuck to the lower surface of the membrane were
123 stained with hematoxylin and counted under a light microscope (Olympus, Japan) in 5 random
124 visual fields (200×). Each experiment was repeated 3 times.

125 **Wound healing assay.** G401 cells were inoculated in 6-well plates. After growing to 95% of
126 confluence, cells were wounded by scratching the vertical lineation using pipette tips, followed by
127 culturing in RPMI 1640 without serum. It was suggested to perform the wound healing assay under
128 without serum and under 10% serum to confirm that the observed effect was caused by the cell
129 migration and proliferation or by cell migration only. Subsequently, cells were treated with miR-
130 200c-3p mimic, mimic NC, miR-200c-3p inhibitor or NC inhibitor, respectively. The distance of
131 the scratch and migrated rate were observed at 0, 24, 48 and 72 h using a light microscope
132 (Olympus, Japan).

133 **Tumor xenograft.** Four-week-old athymic BALB/c nu/nu mice were purchased from the Central
134 Laboratory of Animal Science at Southern Medical University (Guangzhou, China). The mice were
135 housed in our laboratory in a specific pathogen-free environment. To establish the tumor xenograft

136 mice, 1×10^7 SK-NEP-1 cells were injected subcutaneously into right flank of nude mice. Mice
137 (n=4, each group) were exposed with saline (Blank group), Mimic NC (Mock group), miR-200c-3p
138 mimic, and miR-200c-3p inhibitor via intraperitoneal injection. Tumor lengths (L) and widths (W)
139 were measured from day 5 to day 28 using a digital caliper, and tumor volumes were calculated
140 using the equation volume (mm^3)= $L \times W^2/2$. Meanwhile, *in vivo* fluorescence imaging on the IVIS
141 Platform (720p) (PerkinElmer, USA) was performed at 14 and 28 days after transplantation. All
142 animal experiments were conducted in strict accordance with the principles and procedures
143 approved by the Committee on the Ethics of Animal experiments of Southern Medical University,
144 China.

145 **Histological evaluations.** The histological alterations in the tumor tissues were evaluated by the
146 Haematoxylin and Eosin (H&E) (PH0516, Phygene, China) staining. Briefly, tumor tissues were
147 fixed with 4% paraformaldehyde and embedded in routine paraffin. After being dewaxed and
148 hydrated, the renal tissue slides were subjected to H&E staining following standard procedures.
149 Lastly, the sections were observed under a light microscope (Olympus, Japan).

150 **Quantitative real-time PCR (qRT-PCR).** Total RNA, including miRNA from the tissue samples
151 and cultured cells was extracted using Trizol reagent (Invitrogen, USA) according to the
152 manufacturer's instructions. The level of miR-200c-3p was analyzed by qRT-PCR using All-in-One
153 miRNA qRT-PCR Detection Kit (GeneCopeia, USA). cDNA was synthesized from total RNA
154 using TaKaRa reverse transcription kit (TaKaRa, China), and real-time PCR was performed for the
155 detection of miR-200c-3p and EP300. The miR-200c-3p specific forward primer: 5'-
156 GGATAATACTGCCGGGT -3'; specific reverse primer: 5'-GTGCGTGTCGTGGAGTC-3', and
157 the fragment length was 214bp; U6 was amplified as an internal control. The U6 specific forward
158 primer: 5'-GCTTCGGCAGCACATATACTAAAAT-3'; specific reverse primer: 5'-CGCTTCACG
159 AATTTGCGTGTCAT-3' (also used as the reverse primer), and the fragment length was 163bp.
160 The relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method. All experiments were performed in
161 triplicate.

162 **Luciferase activity assay.** The polymerase chain reaction (PCR) was performed to amplify the
163 3'UTR region of the EP300 gene which the miR-200c-3p binding and then the product of PCR was
164 inserted into the vector. A mutant construct in two miR-200c-3p binding sites of EP300 3'UTR
165 region also was generated using Quick Change Site-Directed Mutagenesis Kit (Agilent
166 Technologies, USA). Co-transfections of EP300 3'UTR or mutEP300 3'UTR plasmid with miR-
167 200c-3p lentivirus vector into the cells were accomplished using Lipofectamine^{TM} 3000 (Thermo

168 Fisher Scientific, USA). Luciferase activity was measured using the Dual-Luciferase Reporter
169 Assay System (Promega, USA) after 48 h transfection. Each assay was repeated in 3 independent
170 experiments.

171 **Western blotting analysis.** The RIPA buffer (1× phosphate-buffered saline, 1% NP40, 0.1%
172 sodium dodecyl sulfate, 5mmol/l EDTA, 0.5% sodium deoxycholate, and 1 mmol/l sodium
173 orthovanadate) and protease inhibitors were used to extract protein from cells, and quantified by
174 BCA protein assay kit (#P0012, Beyotime, China). 50 µg protein lysates were resolved on 10%
175 sodium dodecyl sulfate-polyacrylamide by gel electrophoresis and electro transferred to
176 polyvinylidene fluoride (PVDF) membranes (Immobilon P, Millipore, USA). The membranes were
177 blocked with 5% non-fat milk solution for 1 h, and incubated with anti-EP300 antibody (1:1,000,
178 cat. no.70088, CST, USA), anti-Akt antibody (1:1,000, cat. no.4691, CST, USA), anti-p-Akt
179 antibody (1:2,000, cat. no.4060, CST, USA), anti-FOXO1 antibody (1:1,000, cat. no.2880, CST,
180 USA), anti-p-FOXO1 antibody (1:1,000, cat. no.84192, CST, USA) and p27 antibody (1:1,000, cat.
181 no.3688, CST, USA) overnight at 4°C. After Tris Buffered Saline Tween (TBST) washing, the
182 membranes were followed by horseradish-peroxidase-conjugated secondary antibodies (cat.
183 no.5571, CST, USA) and the signal was detected using an enhanced chemiluminescence reaction
184 performed according to the manufacturer's instructions (Alpha Innotech, CA, USA). Image density
185 of immunoblotting was determined by gel densitometry (Bio-Rad, PA, USA). The experiments
186 were independently repeated in triplicates.

187 **Chromatin immunoprecipitation (ChIP) assay.** To further investigate the mechanism of miR-
188 200c-3p involved in the cell proliferation and migration of nephroblastoma, ChIP assay was used to
189 evaluate the relationship between EP300 and P27. Briefly, cells were collected and treated with 1%
190 formaldehyde to perform the cross-linking of protein and chromatin. Then, 0.125 M glycine
191 terminated the cross-linking on ice. After cell disruption by ultrasonic oscillation, the chromatin
192 DNA with the short fragments was extracted and reacted with EP300 antibody(cat. no.12281, CST,
193 USA; IP_group) or IgG (cat. no.2729, CST, USA; negative control), followed by incubation with
194 protein A/G beads, and decrosslinking with proteinase K. Lastly, DNA were extracted to perform
195 qRT-PCR based on the primers that were designed according to the 3 binding site between EP300
196 promoter and P27 (Table 1).

197 **Statistical analysis.** All statistical analyses were performed using SPSS 20.0 statistical software
198 and were calculated from 3 independent experiments. Differences between sample groups and

199 within each sample group were analyzed by one-way ANOVA or by independent-samples t test.
200 Quantitative values are expressed as the mean $\pm$ SD and p-value of < 0.05 was considered
201 statistically significant in all experiment.

202

203 **Results**

204 **Downregulation of miR-200c-3p in nephroblastoma tissues and cells.** To identify the miRNAs
205 that regulate the proliferation and invasion in nephroblastoma, we performed miRNAs array on
206 nephroblastoma tissues and adjacent non-neoplastic renal tissues (n=3/group). The Figure 1A
207 showed the differentially regulated miRNAs between the nephroblastoma tissues and adjacent non-
208 neoplastic renal tissues. From the list of differentially expressed miRNAs (Supplementary Table
209 S1), we found that miR-200c-3p was one of the most significantly downregulated miRNAs in
210 nephroblastoma tissues. However, the underlying mechanism by which the miR-200c-3p in
211 proliferation and invasion in nephroblastoma remain unclear. Firstly, we performed real-time PCR
212 analyses to test miR-200c-3p expression levels in 22 paired fresh nephroblastoma tissues and
213 adjacent non-neoplastic renal tissues. Results showed that miR-200c-3p level in the nephroblastoma
214 tissues was significantly lower than that in the adjacent non-neoplastic renal tissues (p < 0.01,
215 Figures 1B, 1C). Moreover, real-time PCR analyses were also used to explore the miR-200c-3p
216 expression levels in nephroblastoma cells (G401 and SK-NEP-1 cells) and human embryonic
217 kidney cells (293FT cells). We found that miR-200c-3p expression in G401 cells and SK-NEP-1
218 cells was significantly lower than that in 293FT cells (p < 0.05, Figure 1D). These results indicated
219 that miR-200c-3p was downregulated in nephroblastoma, and might be participated in
220 nephroblastoma proliferation and invasion.

221 **EP300 is a direct target of miR-200c-3p in nephroblastoma.** To identify target genes of miR-
222 200c-3p, we used three mRNA target-predicting algorithms, including
223 TarBase(<http://carolina.imis.athena-innovation.gr>), miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw>),
224 miRDB (<http://www.mirdb.org/>) and based on the presence of binding sites in the 3'UTR region.
225 We found that the mRNA levels of 9 candidate target genes, including *AKT2*, *CRKL*, *CBL*, *EP300*,
226 *LAMC1*, *PTEN*, *E2F3*, *CCNE2*, and *CDK2*, were obviously up-regulated in G401 cells and SK-
227 NEP-1 cells compared with 293T cells (p < 0.05, Table 2). Then the miR-200c-3p mimic
228 experiments showed that compared with G401 cells transfected with mimic NC, the mRNA levels

229 of 5 candidate target genes, including *AKT2*, *CRKL*, *CBL*, *EP300*, and *CCNE2*, were remarkably
230 down-regulated in cells transfected with miR-200c-3p mimic ($p < 0.001$, Table 3). To explore the
231 direct relationship between miR-200c-3p and these 5 candidate target genes, we cloned the wide
232 type (WT) 3'UTR or mutation (Mut) 3'UTR of these 5 genes into a dual-luciferase UTR vector.
233 Notably, miR-200c-3p repressed 3'UTR of EP300 and CCNE2 in G401 cells, but had no effect on
234 the activity of Mut EP300 3'UTR ($p < 0.05$, Figures 2A, 2B) and Mut CCNE2 3'UTR
235 (Supplementary Figure S1A). However, dual-luciferase assay showed that miR-200c-3p could not
236 bind to 3'UTR of AKT2, CRKL, and CBL (Supplementary Figure S1A). Furthermore, with miR-
237 200c-3p inhibitor treatment in SK-NEP-1 and G401 cells, we found that the protein levels of
238 endogenous EP300 and CCNE2 were increased. In contrast, ectopic expression of miR-200c-3p
239 significantly reduced the protein levels of endogenous EP300 and CCNE2 in SK-NEP-1 and G401
240 cells ($p < 0.05$, Figure 2C, Supplementary Figure S1B). Notably, EP300 can enter the nucleus and
241 bind to enhancers or promoters, thereby greatly facilitating transcription initiation and activation
242 [13]. Thus, in this study, EP300 was selected as the target gene of miR-200c-3p for the following
243 experiments.

244 **miR-200c-3p inhibits proliferation of nephroblastoma cells.** To further investigate the biological
245 effect of miR-200c-3p in nephroblastoma cells, SK-NEP-1 and G401 cells were treated with miR-
246 200c-3p mimic or inhibitor. CCK8 assay showed that miR-200c-3p mimic significantly inhibited
247 the growth rate of SK-NEP-1 and G401 cells compared with blank group (Figure 3A). Colony
248 formation assay showed that overexpression of miR-200c-3p decreased the colony number of G401
249 cells (Figure 3B). Since SK-NEP-1 cells are semi suspended cells, colony formation assay was not
250 performed. Similarly, soft agar assay also revealed decreased colony number both in SK-NEP-1 and
251 G401 cells after miR-200c-3p mimic treatment (Figure 4A). Conversely, miR-200c-3p inhibitor
252 yielded the opposite effect on cell proliferation of nephroblastoma cells (Figures 3A, 3B, 4A). In
253 addition, cell cycle assay also found that the miR-200c-3p mimic caused a significantly decreased S
254 phase cell population, and a increased cell population in G0/G1 phase in SK-NEP-1 and G401 cells
255 (Figure 4B). indicating that the miR-200c-3p mimic could elicit G0/G1 phase cell cycle arrest.
256 Conversely, miR-200c-3p inhibitor yielded the opposite effect on cell proliferation of
257 nephroblastoma cells (Figures 3A, 3B, 4A, 4B).

258 **miR-200c-3p inhibits invasion and migration of nephroblastoma cells.** To investigate the role of

259 miR-200c-3p played in invasion and migration of nephroblastoma cells, we performed transwell
260 invasion assay on miR-200c-3p transduced nephroblastoma cells. The result showed that miR-200c-
261 3p mimic significantly decreased the number of invading cells; conversely, miR-200c-3p inhibitor
262 obviously increased the number of invading cells (Figure 5A). Wound healing assay was employed
263 to examine the cells migration, and the result showed that miR-200c-3p mimic suppressed
264 migration of nephroblastoma cells compared to the control cells (Figure 5B). When miR-200c-3p
265 inhibitor treated the G401 cells, miR-200c-3p suppressed migration could be reversed (Figure 5B).
266 Since SK-NEP-1 cells are semi suspended cells, Wound healing assay was not performed. Our
267 results demonstrated that miR-200c-3p can inhibit invasion and migration of nephroblastoma cells.

268 **miR-200c-3p inhibits tumor cell proliferation of nephroblastoma *in vivo*.** Moreover, we also
269 explored the effect of miR-200c-3p on cell proliferation *in vivo*. H&E staining of tumor tissues
270 showed that the tumor cells in the miR-200c-3p mimic group was less than that in the other groups,
271 and necrotic tissue was seldom observed in the tumor tissue, accompanied by inflammatory cell
272 reaction, reduced nuclear division and cell atypia. However, in the Blank group, Mock group and
273 miR-200c-3p inhibitor group, tumor cell proliferation was obvious, but inflammatory response was
274 not obvious (Figure 6A). The subcutaneous tumor volume in the miR-200c-3p mimic group was
275 significantly decreased compared to the blank or mock group. And when using miR-200c-3p
276 inhibitor treatment, the tumor volume was drastically increased (Figure 6B). Moreover, *in vivo*
277 fluorescence imaging revealed that tumor formation was not obvious in nude mice of miR-200c-3p
278 mimic group at 14 days compared with the Mock group. However, all nude mice in the Mock and
279 miR-200c-3p inhibitor groups showed significant subcutaneous neoplasms 14 days after
280 transplantation (Figure 6C). On 28 days after transplantation, tumor was formed in nude mice of
281 miR-200c-3p mimic group, and all nude mice in the Mock and miR-200c-3p inhibitor groups
282 showed enlarged subcutaneous neoplasms compared that at 14 days (Figure 6C). Thereby our
283 results revealed that miR-200c-3p inhibited the tumor growth of nephroblastoma.

284 **miR-200c-3p is involved in AKT/FOXO1/p27 signaling pathways in nephroblastoma cells.**
285 Numerous studies have shown that miR-200c-3p suppressed proliferation and invasion by
286 inactivating the AKT pathway in some human carcinomas [14, 15]. In our study, we analysed
287 whether miR-200c-3p participates in the proliferation and invasion of nephroblastoma by activating
288 or inhibiting AKT/FOXO1 pathway. The upregulation of miR-200c-3p markedly suppressed the

phosphorylation of AKT, and enhanced phosphorylation of FOXO1 and p27 expression compared to both the blank and mock groups (Figure 7A). Conversely, inhibiting miR-200c-3p demonstrated a reverse effect. No changes in the total protein levels of AKT and FOXO1 were observed under any these conditions (Figure 7A). To detect whether p27 bind to EP300 promoter, we performed ChIP assay immunoprecipitated with anti-p27 antibody. The result revealed that the positive bands based on the 3 binding sites were successfully amplified by qRT-PCR in the IP group but not in the IgG group (Figure 7B), which suggested that the transcription factor could bind into the EP300 promoter. These results indicated that miR-200c-3p regulate the AKT/FOXO1 pathway via EP300 to play a role in nephroblastoma.

Discussion

According to gene expression regulation reports, it is widely recognized that miRNAs can decrease gene expression by binding the 3'-UTR of their target genes and participate into many key biological processes of cancer progression, such as tumor initiation, cell proliferation and metastasis [16]. MiR-200c is the predominant member of the miR-200 family, which suppresses epithelial-mesenchymal transition, invasion and metastasis in multiple cancers [17, 18]. The significance of miR-200c-3p in cancer biology is more and more important. Such as miR-200 family members were shown to negatively regulate tumour metabolism and progression by targeting lactate dehydrogenase A. MiR-200c-3p played a tumor suppressive role through inhibited High mobility group box 3 (HMGB3) in glioblastoma multiforme [19]. Another study showed that miR-200c-3p tumor suppressor function might contrast with the induction of PD-L1, β -catenin and c-Myc expression by paclitaxel/carboplatin, olaparib and radiation treatments in epithelial ovarian cancer [20]. Evidence had shown that upregulated miR-200c-3p inhibited expression of SOX2, thereby inhibiting development of renal cell carcinoma cells via modulating the Wnt/ β -catenin signaling pathway activation [21]. However, the data about the role of miR-200c-3p in nephroblastoma was rarely published. Therefore, we examined its mechanism of action in the proliferation and invasion of nephroblastoma cells.

Histone acetyltransferase E1A-binding protein p300 (EP300) is a ubiquitously expressed pleiotropic multidomain coactivator that catalyzes acetylation of histone and nonhistone proteins [22]. EP300 mutations contribute to an unfavorable phenotype in a number of solid tumors and hematological

319 malignancies and therefore EP300 is often considered as tumor promoter [23]. Pathological studies
320 have shown that EP300 was highly expressed in many tumors. For example, large breast cancer
321 gene expression data sets revealed that EP300 expression was positively correlated with the
322 expression of cancer stem cells markers and poor prognosis in triple negative breast cancer and
323 basal-like breast cancer [24]. As a mechanism of action, EP300 mutations activated the NOTCH
324 signaling pathway by negatively regulating a key FBXW7(NOTCH repressor), resulting in tumor
325 progression in diffuse large B-cell lymphoma [25]. Esophageal squamous cell carcinoma clinical
326 analyses showed that EP300 promotes epithelial-to-mesenchymal transition process, angiogenesis
327 and hypoxia, and high expression and mutation of EP300 correlates with poor prognosis, thus it
328 may act as an oncogene [26]. EP300, as a transcriptional coactivator, can participate in the
329 regulation of a wide range of biological processes such as proliferation, cell cycle regulation, cell
330 apoptosis and cancer differentiation [27, 28]. Thus, the overexpression of EP300 might contribute to
331 the progression of multiple cancers [29]. However, the mechanism of EP300 in the proliferation and
332 invasion of nephroblastoma is rarely known.

333 Our research investigated the miR-200c-3p-mediated mechanism in nephroblastoma. The
334 expression of miR-200c-3p was significantly lower in nephroblastoma tissues than the adjacent
335 non-neoplastic renal tissues by miRNAs array. Similarly, the expression of miR-200c-3p were
336 lower in G401 and SK-NEP-1 cells than 293FT cells by real-time PCR. The CCK-8 assay, colony
337 formation assay, soft agar assay and cell cycle assay were used to detect proliferation of
338 nephroblastoma cells. We performed transwell invasion assay and wound healing assay to prove
339 invasion and migration of nephroblastoma cells *in vitro*. The tumor xenograft and fluorescence
340 imaging were confirmed the tumor growth *in vivo*. In summary, overexpression of miR-200c-3p
341 inhibited cell proliferation and invasion of nephroblastoma cell , which were consistent with the
342 results reported in other cancer cells [30, 31], suggesting that miR-200c-3p plays an anti-
343 proliferating role in the progression of nephroblastoma. The analysis of bioinformatica databased
344 showed that EP300 maybe a target gene of miR-200c-3p. From the results of mRNA target-
345 predicting algorithms, we proposed that miR-200c-3p might be a novel negative regulator for
346 EP300. Moreover, luciferase activity assay confirmed that miR-200c-3p directly targeted the
347 3'UTR of EP300. Western blot showed that the expression of miR-200c-3p was negative correlated
348 with target gene EP300. Previous studies suggested that EP300 may be involved in oncogenic

349 processes in several different carcinomas including lung, prostate, laryngeal squamous cell cancer,
350 breast cancer and leukemia [32-36]. These results suggested that miR-200c-3p played an anti-
351 tumour role targeting EP300 in nephroblastoma. In addition, in this study, luciferase activity assay
352 confirmed that CCNE2 was also the target gene of miR-200c-3p. Cyclin E2 (CCNE2) has been
353 identified as the second member of E-type CDKs, which contributes to the G1/S phase transition,
354 cell proliferation, tumorigenesis and cancer progression. However, EP300 regulates transcription by
355 serving as scaffolds that bridge sequence-specific DNA binding factors and the basal transcriptional
356 machinery, thereby facilitating transcription through acetylation of histones, transcription factors,
357 and autoacetylation. Finally, EP300 was selected as the research object to explore its signal
358 transduction pathway.

359 It is also well established that EP300 is able to interact with AKT and play a key role in accelerating
360 cancer invasion and metastasis [37]. The AKT/FOXO1 signaling pathway plays a key role in
361 modulating tumor cell proliferation, inhibition of cell cycle, survival and apoptosis in several cancer
362 types [38-41]. We hypothesized that miR-200c-3p might participate in the proliferation and
363 invasion of nephroblastoma by inhibiting the AKT/FOXO1 signaling pathway. FOXO1, a
364 transcription factor that is negatively regulated by AKT-mediated phosphorylation, has been
365 considered to regulate the expression of many genes that are crucial for cell proliferation and
366 invasion. Cyclin dependent kinase inhibitor p27 (also known as Kip1) can regulate the proteolytic
367 degradation and exclusion from the nucleus to inhibit the proliferation of cancers. In quiescent cells,
368 FOXO1 is located in the nucleus in which it can directly bind to the p27 promoter and activate its
369 transcription. In response to oncogenic signals, FOXO1 is phosphorylated by AKT, resulting in
370 nuclear export and retention in the cytoplasm leading to interference in p27 transcription function
371 and thus promoting cell proliferation [42]. Our results validated that miR-200c-3p can decrease the
372 AKT phosphorylation and activation, and leading to high expression and transcriptional activity of
373 FOXO1, which further upregulated the expression of p27. Notably, this study revealed that the
374 transcription factor P27 could bind into the EP300 promoter. In addition, a previous study has
375 demonstrated that the interaction between AKT and EP300 can maintain a steady state level of
376 EP300 and enter the G1 phase of the cell cycle [43]. These results suggested miR-200c-3p can
377 decrease the expression of EP300, and then regulate AKT/FOXO1/p27 signaling pathway to
378 participate in nephroblastoma proliferation and invasion.

379 In summary, our study showed that miR-200c-3p was downregulated in nephroblastoma tissues and
 380 cells and played a key role in the progression of nephroblastoma. Moreover, we found that miR-
 381 200c-3p inhibits the proliferation and invasion of nephroblastoma by targeting EP300 and
 382 inactivating AKT/FOXO1/p27 pathway. The molecular mechanisms explored in this study may
 383 provide new insights and therapeutic strategies for nephroblastoma prevention and treatment.

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388 **Supplementary data are available in the online version of the paper.**

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Figure Legends

Figure 1. miR-200c-3p is downregulated in nephroblastoma tissues and cells. A) heat map of miRNAs expression array in the indicated nephroblastoma tissues (Tumor T1-3) and adjacent non-neoplastic renal tissues (N1-3, n=3/group). B, C) Real-time PCR performed for miR-200c-3p expression in 22 nephroblastoma tissues and corresponding non-neoplastic renal tissues. Data are represented as mean±SD. **p < 0.01 D) MiR-200c-3p expression in nephroblastoma cells. *p < 0.05

Figure 2. EP300 is a bona fide target of miR-200c-3p in nephroblastoma. A) Schematic diagram showing EP300-3'UTR. Sequences were compared between the miR-200c-3p and the wild-type (WT) or mutant (Mut) putative target sites in the 3'UTR of EP300 mRNA. B) Luciferase activities of wild-type 3'UTR EP300-luc and mutant 3'UTR EP300-luc constructs in G401 cells transfected with miR-200c-3p plasmid. Data are represented as mean±SD. *p < 0.05 C) Western blot analysis showing EP300 expression in SK-NEP-1 and G401 cells transfected with miR-200c-3p mimic or

549 miR-200c-3p inhibitor. *** $p < 0.001$

550

551 **Figure 3.** miR-200c-3p suppresses tumor proliferation in nephroblastoma. A) Effect of miR-200c-
552 3p mimic or inhibitor on proliferation of SK-NEP-1 and G401 cells demonstrated by CCK8 assay.
553 Data are represented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$ B) Effects of miR-200c-3p mimic or
554 inhibitor on cell proliferation in G401 cells by colony formation assay. Data are represented as
555 mean $\pm$ SD. * $p < 0.05$

556

557 Figure 4. miR-200c-3p suppresses tumor proliferation in nephroblastoma. A) Effects of miR-200c-
558 3p mimic or inhibitor on cell proliferation in SK-NEP-1 and G401 cells by soft agar assay. Data are
559 represented as mean $\pm$ SD. * $p < 0.05$. B) Effects of miR-200c-3p mimic or inhibitor on cell cycle in
560 SK-NEP-1 and G401 cells. Data are represented as mean $\pm$ SD. * $p < 0.05$

561

562 **Figure 5.** miR-200c-3p suppresses invasion and migration of nephroblastoma cells. A) Effect of
563 miR-200c-3p mimic or inhibitor on the invasion of G401 cells, as tested by transwell assay.
564 Morphologic comparison of cells penetrating the artificial basement membrane is shown. Data are
565 represented as mean $\pm$ SD. * $p < 0.05$. B) Effects of miR-200c-3p mimic or inhibitor on cell
566 migration of G401 cells demonstrated using wound healing assay. Data are represented as
567 mean $\pm$ SD. * $p < 0.05$

568

569 **Figure 6.** miR-200c-3p suppresses tumor growth of nephroblastoma. Subcutaneous tumors of nude
570 mice injected with miR-200c-3p mimic or inhibitor. A) The pathological changes of mice with
571 different groups by Hematoxylin-eosin (HE) staining. B) The nodular volume of subcutaneous
572 tumors are shown. Data are represented as mean $\pm$ SD. * $p < 0.05$ C) The tumor formation of nude
573 mice with different groups at 14 and 28 days after transplantation by *in vivo* fluorescence imaging.

574

575 **Figure 7.** miR-200c-3p is involved in AKT/FOXO1/p27 signaling pathways in nephroblastoma. A)
576 Western blot analyses of AKT, p-Akt, FOXO1, p-FOXO1 and p27 in the cells as indicated. B) The
577 relationship between EP300 and P27 by chromatin immunoprecipitation assay. Data are represented
578 as mean $\pm$ SD. * $p < 0.05$

579

580 **Table 1.** Primers designed according to the 3 binding site between EP300 promoter and P27 for
581 chromatin immunoprecipitation assay.

Gene	F/R	Primer Sequence	Product
p27KIP site1	F	ACAGGCCACATCAAGGAAAC	206
	R	CCGGAGTCAGGACTAGATGC	
p27KIP site2	F	ATGGCCTTAACTGTGCTTGG	206
	R	AGGAGTAGACGCGGCACTTA	
p27KIP site3	F	GCCATATTGGGCCACTAAAA	245
	R	AGTCTCCGCTGATCAAATGG	
GAPDH	F	GCCCATGTTGGTCTCAAAC	209
GAPDH	R	TGCCAACTGTCTCTGTCCAC	

582
583

584 **Table 2.** The mRNA levels of 9 candidate target genes of miR-200c-3p, including *AKT2*, *CRKL*,
585 *CBL*, *EP300*, *LAMC1*, *PTEN*, *E2F3*, *CCNE2*, and *CDK2*, in G401 cells, SK-NEP-1 cells and 293FT
586 cells.

Genes	293T (mean±SD)	G401 (mean±SD)	SK-NEP-1 (mean±SD)	F-value	p-value
AKT2	1.000±0.023	1.489±0.067	1.810±0.038	93.93	< 0.001**
CRKL	1.000±0.018	0.377±0.018	0.121±0.002	1685.485	< 0.001**
CBL	1.000±0.051	1.306±0.096	1.577±0.036	26.145	0.001**
EP300	1.000±0.013	0.543±0.012	0.402±0.014	1294.406	< 0.001**
LAMC1	1.000±0.003	1.323±0.030	1.705±0.013	5945.717	< 0.001**
PTEN	1.000±0.009	0.180±0.031	0.112±0.005	1008.505	< 0.001**
E2F3	1.000±0.075	1.366±0.221	0.511±0.027	7.47	0.024*
CCNE2	1.000±0.037	0.051±0.010	0.320±0.007	648.155	< 0.001**
CDK2	1.000±0.036	0.238±0.010	0.295±0.001	611.162	< 0.001**

587 *p < 0.05, **p < 0.001

588

589 **Table 3.** The mRNA levels of 9 candidate target genes of miR-200c-3p, including *AKT2*, *CRKL*,
590 *CBL*, *EP300*, *LAMC1*, *PTEN*, *E2F3*, *CCNE2*, and *CDK2*, in G401 cells transfected with miR-200c-
591 3p mimic and mimic NC.

Genes	Mimic NC	miRNA-200c mimic	F-value	p-value
AKT2	1.000±0.075	0.030±0.001	3.978	0.004*
CRKL	1.000±0.030	0.356±0.015	0.016	0.000**
CBL	1.000±0.016	0.427±0.011	0.03	0.000**
EP300	1.000±0.037	0.460±0.035	0.351	0.001**
CCNE2	1.000±0.051	0.342±0.032	0.013	0.001**
PTEN	1.000±0.056	0.957±0.031	0.53	0.645
E2F3	1.000±0.057	0.804±0.013	0.003	0.019*
LAMC1	1.000±0.027	1.006±0.053	3.039	0.000**
CDK2	1.000±0.025	0.785±0.038	3.276	0.000**

592 *p < 0.05, **p < 0.001

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