Transcription factor Six2 induces a stem cell-like phenotype in renal cell carcinoma cells

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Renal cell carcinoma (RCC) accounts for 2–3% of adult malignant tumors, and the incidence of RCC worldwide has increased by about 2% over the past two decades [1]. The homeobox protein Six2 has been shown to promote the stemness of breast cancer cells and play a role in kidney development, but its involvement in RCC progression has not previously been investigated. Here, we found that six2 expression was significantly increased in RCC tissues and negatively correlated with the overall survival of patients with RCC. In addition, six2 expression exhibited a remarkably higher level relative to that in normal renal cells. Functional experiments showed that six2 knockdown attenuated the stemness of RCC cells, which was evident by decreased spheroid formation ability and stemness marker (sox2 and nanog) expression. Mechanistic studies indicated that Six2 directly bound to the enhancer of sox2, promoting sox2 expression and downstream effector expression of nanog. Furthermore, overexpression of sox2 rescued the inhibitory effects of six2 on the stemness of RCC cells. Notably, six2 expression is positively correlated with sox2 and nanog expression in RCC tissues. Collectively, our results point toward a six2/sox2 axis responsible for RCC cell stemness.

Abbreviations
CSC, cancer stem cell; kd, knockdown; oe, overexpression; OS, overall survival; qPCR, quantitative real-time PCR; RCC, renal cell carcinoma; six2-kd, kd lentivirus vector for six2.
the progenitor maintenance of nephron progenitors [9,10], and six2 is activated in renal neoplasms and influences cellular proliferation and migration [11]. Because the functional traits between progenitors and CSCs are similar, six2 has been shown to promote the stemness of breast cancer cells [12,13]. However, the roles of six2 in RCC tumor progression are still unclear.

In this study, we showed that six2 expression was significantly increased in RCC tissues and negatively correlated with the overall survival (OS) of patients with RCC. Further functional experiments indicated that kd of six2 attenuated the stemness of RCC cells. Mechanistic studies revealed that Six2 directly bound to the enhancer of sox2, a critical regulator for CSC progression, which is responsible for six2-induced stemness of RCC cells. Our results suggest that six2 may be a potential target for RCC treatments or prognosis.

**Materials and methods**

**Clinical samples and cell lines**

Twenty-six pairs of RCC and normal adjacent paraffin-embedded tissue samples were obtained from the Qianfoshan Hospital of Shandong Province between March 2016 and September 2018. The experiments were undertaken with the understanding and written consent of each subject. Written informed consent from all patients and approval of the hospital ethics review committees were obtained. The study methodologies conformed to the standards set by the Declaration of Helsinki. RCC cell lines SLR23 and SLR20 and human renal epithelial cell line HREpiC were purchased from the Chinese Academy of Sciences Cell Bank. All of the cell lines were cultured in 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS (Thermo Fisher Scientific), 80 U mL⁻¹ penicillin and 0.08 mg mL⁻¹ streptomycin under humified atmosphere with 5% CO₂ at 37 °C.

**Data mining**

The r2: Genomics Analysis and Visualization Platform (http://r2.amc.nl) was used for analyzing the correlation between six2 expression and the OS of patients with RCC. In this platform, the only two kidney tumor datasets, Tumor Kidney Renal Clear Cell Carcinoma (The Cancer Genome Atlas; n = 533) and Tumor Renal Papillary Cell Carcinoma (The Cancer Genome Atlas; n = 290), were used for analysis, and the minimum group size was six (Gene ID: 10736).

**Lentivirus package**

The lentivirus package was constructed by Shanghai Genechem Co., Ltd. (Shanghai, China). The kd lentivirus vector for six2 and overexpression (oe) lentivirus vector for sox2 were designated six2-kd and sox2-oe, respectively. In addition, control empty lentivirus vector served as a control group in this work. The stable cell lines with six2-kd were screened by culturing with puromycin.

**Quantitative real-time PCR**

Total RNAs from tissues and cells were extracted with TRIeasyTM Total RNA Extraction Reagent TRIeasyTM (YEASEN, Shanghai, China) following the manufacturer’s recommendation. Then, cDNA was reversely synthesized using HiScript® Q Select RT SuperMix for quantitative real-time PCR (qPCR; Vazyme, Nanjing, China) according to the standard procedure. Quantitative RT-PCR was performed on the StepOne Plus PCR system with AccQ® Universal SYBR® qPCR Master Mix (Vazyme). Glycerolaldehyde-3 phosphate dehydrogenase served as an internal reference. The relative expression levels of transcripts were calculated using the 2⁻ΔΔct method.

**Western blot**

The detailed procedure was described previously [14]. The primary antibodies against these proteins were used: six2 (1 : 1000; Wanleibio, Shenyang, China), nanog (1 : 1000; Proteintech, Wuhan, China) and β-actin (1 : 5000; Beyotime, Beijing, China).

**Spheroid formation assay**

The detailed procedure was mentioned in the previous work [15]. In brief, RCC cells were cultured in ultra-low attachment 24-well plates (Corning, Union City, CA, USA) at 1000 cells per well with Dulbecco’s modified Eagle’s medium/F12 medium supplemented with 1× B27 (Sigma, St. Louis, MO, USA), 20 ng mL⁻¹ basic fibroblast growth factor (Sigma), 20 ng mL⁻¹ epidermal growth factor (Sigma), 4 μg mL⁻¹ heparin (Sigma) and antibiotics at 37 °C under a 5% humidified CO₂ atmosphere. After 10 days, the number and size of spheroid were evaluated and quantified under a microscope.

**Luciferase reporter assay**

Sox2 DNA contains two evolutionally conserved enhancers, SRR1 and SRR2. The SRR2 domain was inserted into the pGL3-Enhancer vector, named pGL3-sox2. The empty vector served as a control vector. In addition, we constructed a single point mutation (T27G) within the putative six2 binding region of the SRR2 enhancer based on a six2 binding site that was previously identified via Mut Express MultiS Fast Mutagenesis Kit (Vazyme) [16], named pGL3-sox2-T27G. The pGL3-sox2, pGL3-sox2-T27G or empty vector and Renilla luciferase construct were cotransfected into RCC cells with six2 stable oe using Lipofectamine 3000 (Thermo Fisher Scientific) and the recommendation. Then, cDNA was reversely synthesized using HiScript® Q Select RT SuperMix for quantitative real-time PCR (qPCR; Vazyme, Nanjing, China) according to the standard procedure. Quantitative RT-PCR was performed on the StepOne Plus PCR system with AccQ® Universal SYBR® qPCR Master Mix (Vazyme). Glycerolaldehyde-3 phosphate dehydrogenase served as an internal reference. The relative expression levels of transcripts were calculated using the 2⁻ΔΔct method.
Fisher Scientific) following the manufacturer’s recommendation. Seventy-two hours later, cell lysates were collected, and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The activity of *Renilla* luciferase was used for normalization.

**ChIP-qPCR**

ChIP-qPCR analysis was performed using the ChIP kit (Cat No. ab500; Abcam, Cambridge, MA, USA) according to the standard procedure. DNA produced using this kit was used for detecting the enrichment of SRR2 of sox2 by qPCR assay. The ChIP antibody for sox2 and relative negative antibody were purchased from Proteintech (Cat No. 11562-1-AP).

**Cell viability**

Cell viability was assessed by the Cell Counting Kit-8 (Gilson, Middleton, WI, USA) assay. Cells with different treatments were plated in a 96-well plate at the density of 3000 cells per well and incubated for 24, 48 and 72 h. The number of viable cells was evaluated by the Cell Counting Kit-8, and 600 nm absorbance was measured using a Microplate Reader (BIO-TEK, Winooski, VT, USA). All values were normalized to the R600 value of the control group, which was regarded as 1.

**Statistical analysis**

Data analysis was carried out using GRAPHPAD PRISM 6 (Version X; La Jolla, CA, USA). Standard error mean calculations were used for generating error bars. An unpaired one-tailed Student’s *t*-test was performed to assay the significance between two groups. One-way ANOVA followed by a Bonferroni comparison was used for experiments with three or more conditions. Asterisks represent the significance of difference from the control group: *P* < 0.05, **P** < 0.01.

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**Fig. 1.** Six2 expression is significantly increased in RCC tissues and cells, and negatively correlated with the OS of patients with RCC. (A) Six2 mRNA level was examined in RCC and normal adjacent tissues by qPCR assay. (B) Six2 mRNA level was detected in RCC and normal renal epithelial cells. (C) Six2 protein level was measured in RCC and normal renal epithelial cells. (D, E) The correlation between six2 expression and the OS of patients with RCC was evaluated in two datasets from the R2 platform. The difference was assayed using one-way ANOVA with the Tukey-Kramer post-test. Data were presented as the mean ± SD. **P** < 0.01 versus normal adjacent tissues or HREpiC. bonf, Bonferroni; grp, group; min., minimum; TCGA, The Cancer Genome Atlas.
Results

**Six2 expression is significantly increased in RCC tissues and cells, and negatively correlated with the OS of patients with RCC**

First, we detected *six2* expression in RCC and normal adjacent tissues, and found that *six2* was significantly increased in RCC tissues compared with that in normal adjacent tissues (Fig. 1A). In agreement, *six2* displayed a higher level in RCC cells relative to the normal renal epithelial cells (Fig. 1B,C). Notably, analysis on the two datasets in the R2 platform showed that *six2* expression was negatively correlated with the OS of patients with RCC (Fig. 1D,E). These results indicate that *six2* may hold promoting roles in RCC progression.
Six2-kd attenuates the stemness of RCC cells

Because CSCs contribute to tumor progression, recurrence and metastasis, we wondered whether six2 facilitates the stemness of RCC cells. First, we detected the six2 level in nonadherent RCC spheroid cells and found that six2 exhibited a remarkably higher level relative to the adherent cells (Fig. 2A,B). Because six2 expression was higher in RCC cells and tissues, we chose to knock down six2 expression in RCC cells by lentivirus infection, and stable cell line construction, qPCR and western blot confirmed the kd efficiency of six2-kd (Fig. 2C,D). Notably, six2-kd had no effects on RCC cell viability (Fig. 2E). As expected, six2-kd reduced the expression of stemness regulators (sox2 and nanog; Fig. 2F,G). In addition, spheroid formation ability was attenuated by six2-kd, characterized as the decrease of spheroid size and number (Fig. 2H,I). Therefore, these results demonstrate that six2 positively regulates the stemness of RCC cells.

Six2 directly binds to the SRR2 domain of sox2 and thus promotes its transcriptional activity

Then, we explored the mechanism by which six2 regulates the stemness of RCC cells. Two recent studies showed that six2 promotes the stemness of breast cancer cells via different mechanisms; one indicated that six2 activates the competing endogenous RNA network between CYP4Z1 and pseudogene CYP4Z2P via directly binding to their promoters [17], and another work demonstrated that Six2 directly binds to the sox2 SRR2 enhancer region [18]. Because CYP4Z1 and
pseudogene CYP4Z2P were not expressed in RCC [19], we assumed that Six2 might promote the stemness of RCC cells via directly binding to the sox2 SRR2 enhancer region. As expected, the basal pGL3-sox2 activity was higher in RCC cells than that in normal HREpiC cells, and six2-kd reduced the luciferase activity of pGL3-sox2 in RCC cells, whereas the activity of pGL3-sox2-T27G was unaffected (Fig. 3A–C). Further, we performed ChIP-qPCR and found that the SRR2 region was enriched in DNA pulled down by anti-Six2, whereas six2-kd reduced the SRR2 enrichment (Fig. 3D). To further specify the binding region of SRR2, we performed ChIP-qPCR to analyze the enrichment of a region approximately 5 kb downstream of the SRR2 region, where no Six2 binding sites exist. As shown in Fig. 3E, the enrichment of Six2 observed in the SRR2 region was lost. Taken together, these results suggest that Six2 indeed directly binds to the SRR2 region of sox2 in RCC cells.

**Six2 regulates the stemness of RCC cells dependent on sox2**

We further investigated whether six2 regulates the stemness of RCC cells through sox2. Sox2 was overexpressed in RCC cells with six2 stable kd via lentivirus infection, and the infection efficiency was confirmed by western blot and quantitative RT-PCR (Fig. 4A,B). As expected, the decreased expression of another stemness master regulator nanog was rescued by sox2-oe (Fig. 4C,D). In addition, six2-kd-mediated decrease of spheroid formation ability was partially reversed by sox2-oe (Fig. 4E,F). Collectively, our results demonstrate that six2 positively regulates the stemness of RCC cells in a sox2-dependent manner.

**Six2 exhibits a positive correlation with the stemness markers**

Finally, to further confirm our conclusion, we detected the correlation between six2, sox2 and nanog expression in clinical samples and online datasets from the r2 platform. We found that the expression of six2 and sox2 or nanog exhibited a positive correlation in clinical RCC and normal tissues (Fig. 5A–D). Notably, a consistent result was observed in the online dataset from the r2 platform (Fig. 5E–H).

**Discussion**

This work indicated that transcription factor Six2 positively regulated the stemness of RCC cells via directly binding to the SRR2 enhancer of stemness master regulator sox2. Although the promoting roles of six2 have
been established in other tumors, to our knowledge, this is the first work to reveal the roles of six2 in RCC cell stemness.

CSCs have been regarded as the root of tumor progression, recurrence, metastasis and drug resistance. A previous study showed that salinomycin exhibits a specific killing capacity on CSCs by sequestering iron in lysosomes [20]. In addition, salinomycin could suppress the stemness and induce the apoptosis on human ovarian CSCs [21]. Notably, salinomycin reduces the stemness and epithelial-mesenchymal transition process of RCC cells [22]. Moreover, Zheng et al. [23] showed that a CD13-targeting peptide integrated protein inhibits human liver cancer growth by killing CSCs. These results suggest that there are drugs that specifically kill CSCs; this should be further explored in the future. In the present study, we found that transcriptional factor six2 positively regulates the stemness of RCC cells.

Fig. 5. Six2 exhibits a positive correlation with the stemness markers. (A) The correlation between six2 and sox2 expression was evaluated in RCC tissues. (B) The correlation between six2 and nanog expression was examined in RCC tissues. (C, D) The correlation between six2, sox2 and nanog expression was determined in normal tissues. (E, F) The correlation between six2 and sox2 expression was determined in two RCC datasets from the r2 platform. (G, H) The correlation between six2 and nanog expression was analyzed in two RCC datasets from the R2 platform. TCGA, The Cancer Genome Atlas.
Evaluating the previous works that reveal that six2 promotes the stemness of breast cancer cells [17,18], we wonder whether the promoting roles of six2 on cancer cell stemness are common in other tumors. Future work should be performed to resolve this issue, which could facilitate six2 as the drug target.

Notably, the previous study has shown that nanog acts as the downstream effector of sox2 in breast cancer [24]; this result is consistent with our results in this work. In addition, the roles of six2 in nephrogenesis, coupled with six2 expression in RCC, indicate that six2 may be a critical regulator of both renal cell stemness and RCC stemness, and it will be of interest to determine whether six2 similarly regulates sox2 and the downstream effector nanog in the context of normal renal development. Importantly, we must admit that six2, like sox2, is a transcriptional factor and thus not an ideal drug target. Collectively, this work identifies that six2 plays a critical role in promoting RCC cell stemness through six2/sox2 signaling. Notably, the relationship between six2 and sox2 or nanog expression is positively relevant, and six2 expression is negatively correlated with the OS of patients with RCC. Therefore, it will be important to identify more targetable downstream effectors of six2 that are critical in mediating its ability to promote RCC cell stemness.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

NC, HL, YH and SS conceived and designed the project. NC, HL and YH acquired the data. NC and HL analyzed and interpreted the data. NC and HL wrote the paper.

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