SOX2 Maintains the Stemness of Retinoblastoma Stem-like Cells Through Hippo/YAP Signaling Pathway

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Abstract

Background: Cancer stem cells are responsible for tumor initiation and progression in various types of cancer, while, although the existence of retinoblastoma stem cells had been reported, the mechanisms supporting retinoblastoma stemness are poorly understood. In this study, a modified method for isolating retinoblastoma stem-like cells for mechanistic study was first established and an important mechanism underlying SOX2-drived retinoblastoma stemness was subsequently revealed.

Methods: The retinoblastoma stem-like cells were isolated by single cell cloning in combination of examination of sphere-forming capacities. The stemness of isolated retinoblastoma stem-like cells were characterized by sphere-forming capacities and the expression of cancer stem cell markers. The SOX2 gene was overexpressed or knocked down by lentivirus system. The transcriptional regulation was identified by qRT-PCR, luciferase reporter, nuclear run-on and DNA pull down assay. Spearman analysis was employed for correlation analysis of genes in tumor tissues of retinoblastoma patients.

Results: The isolated retinoblastoma stem-like cells exhibited significantly enhanced sphere-forming capacity and constantly higher levels of CD44, ABCG2, SOX2 and PAX6, but not CD133. SOX2 positively regulated the stemness of retinoblastoma stem-like cells as identified by gene manipulation technology. SOX2 directly binds to the promoters of WWTR1 and YAP1, transcriptionally activates WWTR1 and YAP1, and thereby activating Hippo/YAP signaling. Knockdown of WWTR1 or YAP1 partially abolished the effect of SOX2 on the stemness of retinoblastoma stem-like cells.

Conclusion: An effective method for isolation of retinoblastoma stem-like cells was established. The mechanistic study demonstrated that SOX2, as a key deriver, maintains retinoblastoma stemness by activating Hippo/YAP signaling. Inhibition of Hippo/YAP signaling would be an effective strategy for human retinoblastoma caused by aberrant upregulation of SOX2.

Introduction

Most solid cancer tissues are hierarchically organized, in which a small subpopulation of cells with stem cell properties, named cancer stem cells (CSCs), are primarily responsible for tissue growth and progression, and in contrast, most of differentiated cells are non- or poor-tumorigenic [1]. Targeting cancer stem cells have been recognized as an effective strategy for cancer therapy [2].

Retinoblastoma is the most common eye tumor in children that develops from the immature cells of retina [3]; the incidence of this disease is 1 in 15,000–20,000 live births and the median age at first diagnosis is two years [4]. So far, non-invasive therapeutic strategies for retinoblastoma are limited; current therapeutic methods, including surgery (enucleation) and radiation, usually results in blindness and inactive treatment causes it life-threatening [5].

Retinoblastoma is also characterized by heterogenicity; multiple molecular changes are able to drive retinoblastoma transformation, such as germline mutation of *RB1* gene, aberrant inactivation of RB

protein (encoded by *RB1* gene) and *MYCN* amplification [6]. In addition, retinoblastoma can arise from retinal stem cell, progenitor cell, post-mitotic cell and differentiated cell [7]. Furthermore, retinoblastoma exhibits both intrapatient and intratumoral heterogenicity [7]. These phenomena of heterogenicity suggests the existence of CSCs in retinoblastoma. Indeed, several studies have reported the evidences of retinoblastoma stem cells [8–16], however, the isolation strategies for mechanistic study of retinoblastoma stem cells have not been fully established and the mechanisms underlying the maintenance of the stemness of retinoblastoma stem cells are rarely reported, which limited the development of the therapeutic strategies for eradicating retinoblastoma stem cells.

The objective of this study is to establishment of a feasible isolation strategy of retinoblastoma stem-like cells and to reveal a SOX2-related mechanisms underlying the stemness maintenance of retinoblastoma stem-like cells.

Materials And Methods:

Cell culture

The Y79 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and Weri-RB1 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Both cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (#12633012, Thermo Fisher, Waltham, MA, USA) containing 10% fetal bovine serum (FBS, #16140071, Thermo Fisher, Waltham, MA, USA) and antibiotics (#15640055, Thermo Fisher, Waltham, MA, USA). All the cells were identified by short tandem repeat profiling and validated for absence of mycoplasma.

Primers and antibodies

Primers used in this study were provided in Supplementary Table 1. The antibodies used in this study were listed in Supplementary Table 2.

Isolation of retinoblastoma stem-like cells

The Y79 and WERI-RB1 cells were first normally cultured. When the cells reached 80% confluence, the cells were washed and dissociated. The dissociated cells were then resuspended in stem cell culture medium (RPMI-1640 medium containing 20 ng ml^{-1} recombinant human epidermal growth factor (rhEGF, #PHG0314, Thermo Fisher, Waltham, MA, USA), 20 ng ml^{-1} recombinant human basic fibroblast growth factor (rh-bFGF, #PHG0261, Thermo Fisher, Waltham, MA, USA), 1× B-27 (#17504044, Thermo Fisher, Waltham, MA, USA), 5 µg ml^{-1} heparin (H0200000, Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine (#21051040, Thermo Fisher, Waltham, MA, USA), 20 ng ml^{-1} recombinant human leukemia inhibitory factor (rh-LIF, # PHC9483, Thermo Fisher, Waltham, MA, USA), and antibiotics). The single-cell suspension was then prepared by repetitive pipetting. The cells were then diluted by stem cell medium to a concentration of 1 cell per 10 µl. Then, the cells were seeded into ultra-low attachment 96-well plate (#3474, Corning Inc., Corning, NY, USA) at the density of 1 cell per well (10 µl single-cell suspension

solution per well), following by careful check under microscope. The cells were cultured for 25 days and the spheres in each well were checked under microscope. The cells with the strongest sphere-forming capacity were selected and cultured in ultra-low attachment dish for expansion. The stem-like properties (including sphere-forming capacity, the frequency of sphere-forming cells and the expression of stem cell makers) were subsequentially determined.

Sphere-formation assay

The cells were first cultured in normal culture medium for at least 24 hours. When the cells reached 80% confluence, the cells were washed and single-cell suspension solution was prepared by stem cell culture medium. The cells were then seeded into ultra-low attachment 6-well plate (#3471, Corning Inc., Corning, NY, USA) at the density of 3,000 cells per well. After 15 days of culture, the spheres in each well were counted under microscope.

Real-time quantitative reverse-transcription PCR (qRT-PCR).

Total RNA from indicated cells was isolated with Trizol reagent (#A33254, Thermo Fisher, Waltham, MA, USA). The ethanol was removed through evaporation. The residual DNA was removed by Turbo DNAse (#AM2239, Thermo Fisher, Waltham, MA, USA) according to manual, followed by DNAse inactivation. The mRNA concentration was determined by Nanodrop spectrophotometer (Thermo Fisher, Waltham, MA, USA). TaqMan RNA-to-Ct 1-Step Kit (A25605, Thermo Fisher, Waltham, MA, USA) was used for qRT-PCR assay. *GAPDH* was chosen as internal control. The relative mRNA levels were presented as $\Delta\Delta$Ct. The details of primer are listed in supplementary materials.

Correlation analysis

The gene expression data (Tumor Retinoblastoma – Dorsman – 76 dataset) was downloaded from R2 bioinformatics website (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi). The Spearman correlation analysis was used for determined the correlation between genes.

Isolation of CD44+ and ABCG2+ cells

The CD44+ and ABCG2+ cells were isolated by MACS system (magnetic activated cell sorting) according to manufacturer instruction. All reagents and products related to MACS were purchased from Miltenyi Biotec (Auburn, CA, USA). Briefly, 50 µl of cell suspension (10^7 cells) was mixed with 25 µl of blocking reagent and 25 µl of anti-CD44 (#130-095-194, Miltenyi Biotec, Auburn, CA, USA) or anti-ABCG2 (#130-10-7-680, Miltenyi Biotec, Auburn, CA, USA) microbeads, followed by incubation for 20 min at 4 °C. After centrifugation (1,000 rpm for 5 min), the supernatant with cells were loaded onto a MACS column, the CD44+ or ABCG2+ cells retained in the column and CD44- or ABCG2- cells that passed through column were harvested.

Gene manipulation

Lentivirus vectors pCDH-CMV-MCS-EF1-Puro and PLKO.1-puro were kindly provided by professor Hongbin Ji (Shanghai Institutes for Biological Sciences, Shanghai, China). For SOX2 overexpression, the coding sequence of SOX2 was inserted into pCDH-CMV-MCS-EF1-Puro plasmid. For SOX2, YAP and WWTR1 knockdown, shRNAs specifically against SOX2, YAP and WWTR1 were inserted into PLKO.1-puro plasmids. The shRNAs used were provided in Supplementary Table 3. The lentivirus were packaged by co-transfection with the reconstruction, VSV-G and delta R8.2 plasmids into HEK293T cells. After 24 hours, the medium containing virus particles was collected and used for infection. The stable cell lines were selected by puromycin and the efficiency of transfection was determined by western blot.

Western blot

Western blot was performed according to standard protocol. Briefly, RIPA buffer (#89901, Thermo Fisher, Waltham, MA, USA) was used to prepare the cell lysis. After centrifugation, the protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto Immobilon-P membranes (#IPVH00010, Millipore-Sigma, St. Louis, MO, USA), followed by incubation with the primary antibodies at 4 °C overnight. After incubation with corresponding horseradish peroxidase (HRP)-conjugated secondary antibody, the signals were visualized by incubation with Immun-Star HRP Substrate (#1705040, BioRad, Hercules, CA, USA). β-actin and Lamin B1 were used as internal control for total and nuclear protein, respectively. Uncropped gels were attached in Supplemented Figure 2.

Xenograft model

the cells were harvested and resuspended in the medium containing 25 % Matrigel (A1413301, Thermo Fisher, Waltham, MA, USA), followed by inoculation into the flank of 6-8 weeks old female BALB/c nude mice (Pusheng Technology, Nanjing, China). The tumors were counted at 30-45 days after incubation. All the procedures were approved by Institutional Animal Care and Use Committee in the Affiliated People's Hospital of Ningbo University, (Zhejiang, China).

Limiting dilution assay

The cells were first cultured in 6 cm cell culture dish. When the cell density reached 90% confluence, the cells were harvested and resuspended in sphere medium as mentioned above. The cells were then cultured in 96-well ultra-low attachment culture plate (#3474, Corning Inc., Corning, NY, USA) at a density of 20, 10, 5 cells / well. After 25 days culture, the number of wells with spheres was recorded. To determine the frequency of tumor-initiating cells in vivo, the cells were inoculated into the flank mice at the density of 50, 25, 5 cells per mice. The number of mice with tumor was counted at 45 days after inoculation. ELDA online software (http://bioinf.wehi.edu.au/software/elda/) was used to calculate the frequency of sphere-forming cells.

Chromatin immunoprecipitation quantitative PCR (ChIP-PCR)

EpiTect ChIP qPCR Assay kit (#334211, Qiagen, Valencia, CA, USA) was used for ChIP-PCR assay. Briefly, the cells were fixed for cross-linking, followed by isolation of chromatin. The chromatin DNA was subsequently fractionated to 0.8 to 1.0 kb by sonication, followed by incubation with SOX2 antibody-bound beads. The DNA samples on the bead were then reverse cross-linked and collected. After purification and quantification, the Levels of YAP and WWTR1 promoter were determined by qRT-PCR.

Luciferase reporter assay

To determine the transcriptional activity of YAP and WWTR1 promoters, the promoter fragments were cloned into PGL4.20 luciferase reporter plasmid (E6751, Promega, WI, USA). To analyze the transcriptional activity of YAP, an 8XGTIIC-luciferase reporter plasmid containing YAP/TAZ target sequence was used. The reconstructed plasmids and hRluc/TK control plasmids were co-transfected into the cells. The luciferase activity was determined by Dual-Luciferases Reporter Assay kit (E1901, Promega, WI, USA) according to the manual.

Nuclear run-on assay

The cells were cultured in 6-well plate containing complete culture medium to 70% confluence, followed incubation with medium containing 0.5 mM 5-ethynyl uridine (EU, E10345, Thermo Fisher, Waltham, MA, USA) for 1 h. The total RNA was subsequently isolated. After biotinylation with biotin azide, the labled RNA was purified by streptavidin coupled beads, followed by qRT-PCR examination.

DNA pull-down assay

The bait DNA fragments were labeled with Biotin using 3' End DNA labeling Kit (#89818, Thermo Fisher, Waltham, MA, USA). The labeled DNA fragments were subsequently immobilized on the Dynabeads^{TM} M-270 Streptavidin (#65306, Thermo Fisher, Waltham, MA, USA). The nuclear extracts from 2 × 107 cells were prepared, followed by incubation with bait DNA-coated beads in binding buffer. The DNA-protein complex bound by beads was then eluted and subjected to western blot examination.

Data analysis

Data were represented as 'mean ± SD' from three experiments except where indicated. The significance was determined by Student's t-test and One-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001).

Results:

Establishment and characterization of retinoblastoma stem-like cell

Formation of regular spheres in suspension culture environment is the prime property of CSCs and thus served an ideal characteristic for retinoblastoma stem-like cell selection [17]. As shown in Figure 1A, single cells of the parental retinoblastoma cells (Y79 and Weri-RB1) were seeded into ultra-low attachment 96-well culture plate and maintained in serum-free culture medium to maintain the self-

renewal capacity and to prevent differentiation. After 25 days culture, the cells which formed regular spheres were selected as retinoblastoma stem-like cells. The retinoblastoma stem-like cells were continually cultured in ultra-low attachment dish with serum-free culture medium.

The sphere-forming capacity and the expression of stem cell markers were used to characterize the isolated retinoblastoma stem-like cells. As shown in Figure 1B, the retinoblastoma stem-like cells exhibited significantly enhanced sphere-forming capacity, compared to parental cells, reflected by both increased number and diameter of spheres. As shown in Figure 1C, the expression of surface markers, CD44 and ABCG2, and the transcription factors, SOX2 and PAX6, normally associated with CSCs was significantly upregulated in the isolated retinoblastoma stem-like cells. While, the regulation of surface marker CD133 is not constantly, which was downregulated and upregulated in Y79-derived and Weri-RB1 derived stem-like cells (Figure 1C), respectively. These results demonstrated that retinoblastoma stem-like cells could be screened successfully by single cell cloning in combination of examination of sphere-forming capacities and CD44, ABCG2, SOX2, PAX6 could be served as markers for retinoblastoma stem-like cells

SOX2 positively regulate the stemness of retinoblastoma stem-like cell

SOX2 have been recognized as key player for stemness maintenance in both normal and cancer stem cells [18]. In this study, we aim to reveal the effect of SOX2 on the stemness of retinoblastoma and the underlying mechanism. By bioinformatic study, we found that the expression of SOX2 is positively correlated with CD44, ABCG2 and PAX6 in the tumor tissues of retinoblastoma patients (Figure 2A). Consistent with this result, in isolated CD44+ and ABCG2+ retinoblastoma cells, the mRNA levels of SOX2 were significantly upregulated (Figure 2B). These results indicated that SOX2 may plays important role in maintenance of high expression levels of retinoblastoma stem-like cell markers.

Next, the SOX2 was overexpressed in retinoblastoma stem-like cells. The lentivirus containing SOX2-overexpressing plasmids were used to infect the retinoblastoma cells under normal culture condition, followed by puromycin selection. The SOX2-overexpressing retinoblastoma stem-like cells were then prepared by suspension culture in serum-free medium. As shown in Figure 3A, the protein levels of SOX2 in SOX2-overexpressing cells were significantly upregulated, which demonstrated that the SOX2-overexpressing cells were successfully established. As shown in Figure 3B, SOX2 significantly increased the sphere-forming capacity of retinoblastoma cell. In addition, SOX2 overexpression significantly upregulated the expression of the retinoblastoma stem-like cell markers (CD44 and ABCG2) as identified by qRT-PCR and western blot (Figure 3C and 3D). Moreover, the results from limiting dilution assay showed that SOX2 significantly increased the frequency of sphere-forming cells in vitro (Figure 3E) and the frequency of tumor-initiating cells in vivo (Figure 3F). These results demonstrated that SOX2 promotes the stemness of retinoblastoma stem-like cells.

To confirm this result, we subsequently established SOX2-knockdown retinoblastoma stem-like cells (Figure 4A). As expected, knockdown of SOX2 significantly decreased the stemness of retinoblastoma stem-like cells reflected by attenuated sphere-forming capacity (Figure 4B), the expression of CD44 and

ABCG2 (Figure 4C and 4D), and the frequency of sphere-forming cells in vitro (Figure 4E and Supplementary Figure 1). Collectively, these results demonstrated that SOX2 positively regulates the stemness of retinoblastoma stem-like cells.

SOX2 activates Hippo/YAP signaling in retinoblastoma stem-like cell

Hippo/YAP signaling plays a fundamental role in regulation of the stemness of both normal and cancer stem cells [19]. To investigate the mechanisms underlying SOX2 supporting the stemness of retinoblastoma stem-like cell, we performed the correlation analysis with gene expression data derived from retinoblastoma tissues and found that SOX2 is positively correlated with YAP and WWTR1 (Figure 5A), the prime transcription coactivators in Hippo/YAP signaling pathway [19], which indicates that SOX2 may activate Hippo/YAP signaling. Consistent with this result, SOX2 is positively correlated with several YAP target genes in Hippo/YAP signaling, including CTGF, ITGB4 and FGF2 [19] (Figure 5B), which strengthens the clinical evidences for the positive regulator role of SOX2 in Hippo/YAP signaling.

Next, we tested the expression of YAP target genes in SOX2-overexpressing cells and found that SOX2 significantly increased the expression of YAP target genes, including CTGF, ITGB4 and FGF2, as identified by qRT-PCR (Figure 5C). As the transcriptional activity of YAP largely depends on the protein level of nuclear YAP [19], we next examined the effect of SOX2 on the protein level of YAP in nucleus. As shown in Figure 5D, SOX2 significantly increased the protein level of YAP in nucleus of retinoblastoma stem-like cells. Furthermore, the employed a luciferase reporter plasmid containing the target of YAP to monitor the effect of SOX2 on YAP transcriptional activity. As shown in Figure 5E, SOX2 significantly increased the transcriptional activity of YAP in retinoblastoma stem-like cells. These results demonstrated that SOX2 activates Hippo/YAP signaling in retinoblastoma stem-like cells.

SOX2 transcriptionally activates YAP and WWTR1 in retinoblastoma stem-like cell

As the prime function of SOX2 in mammalian cells is transcription factor [18], we speculated that SOX2 directly binds to YAP and WWTR1 promoters and transcriptionally activate YAP and WWTR1. We first performed qRT-PCR assay and found that SOX2 significantly increased the mRNA levels of YAP and WWTR1 in retinoblastoma stem-like cells (Figure 6A). To confirm the effect of SOX2 on the transcription of YAP and WWTR1, the nuclear run-on assay was performed. As shown in Figure 6B, SOX2 significantly increased the nascent transcripts of YAP and WWTR1 genes in retinoblastoma stem-like cells. Moreover, the results from luciferase reporter assay showed that SOX2 significantly increased the transcriptional activity of YAP and WWTR1 promoters, and 500-1000 base pairs upstream from the start site of transcription is critical for this process of both genes (Figure 6C). These results demonstrated that SOX2 promotes the transcription of YAP and WWTR1.

Next, to demonstrate the direct bindings between SOX2 and YAP and WWTR1 promoters, ChIP-PCR and DNA pull-down assay were performed. As shown in Figure 6D, the promoters of YAP and WWTR1 were significantly immunoprecipitated by SOX2 antibody. Moreover, the SOX2 proteins were also significantly eluted from beads reacted with YAP and WWTR1 promoters (Figure 6E), which demonstrated the direct

bindings between SOX2 and YAP and WWTR1 promoters in retinoblastoma stem-like cells. Taken together, these results demonstrated that SOX2 directly bind to YAP and WWTR1 promoters and promotes their transcription.

Upregulation of YAP and WWTR1 is necessary for effect of SOX2 on the stemness of retinoblastoma stem-like cell

To confirm that SOX2-induced upregulation of YAP and WWTR1 is the main mechanism underlying SOX2 supporting the stemness of retinoblastoma stem-like cell, the YAP and WWTR1 were knocked down in SOX2-overexpressing cells (Figure 7A). As expected, knockdown of YAP or WWTR1 significantly abolished the stimulatory effect of SOX2 on sphere-forming capacity (Figure 7B) and the expression of stem cell maker expression (Figure 7C) and YAP target genes (Figure 7D), which demonstrated that upregulation of YAP and WWTR1 is critical for the stimulatory effect of SOX2 on the stemness and Hippo/YAP signaling in retinoblastoma stem-like cell.

Discussion

In this study, we developed a method for isolation of retinoblastoma stem-like cells. Furthermore, the SOX2-related mechanism underlying stemness maintenance of retinoblastoma stem-like cells was revealed.

Non-invasive therapeutic methods targeting retinoblastoma stem cells are important for retinoblastoma patients; invasive therapeutics, eye removal surgery for example, causes blindness and unfavorable side effects are difficult to be avoid for radiation therapy [20]. Although several chemotherapeutic drugs, such as carboplatin and doxorubicin, were used for retinoblastoma [21], these drugs are not developed for targeting cancer stem cell, which is the root of cancer responsible for prime tumor initiation and progression.

The evidences of the existence of retinoblastoma stem cells have been reported in several studies. G.M.Seigel and colleagues detected retinoblastoma stem cells base on stem cell makers, such as ABCG2 [9, 10]. Zhong x et al. isolated retinoblastoma stem cells in human solid tumor [11]. Differentiation experiments with retinoblastoma stem cells have been performed in the studies from Hu hu and colleagues [12]. Importantly, the results form Zhixin Zhang and co-workers' study showed that the rate of retinoblastoma stem cells in Y79 cell line is appallingly low [13], compared to primary cells, which indicates the necessity of single cell cloning in isolation of retinoblastoma cells. In this study, the retinoblastoma stem-like cells derived from single cell clones with conspicuous sphere-forming capacity formed regular spheres in serum-free culture medium and consistently expressed stem cell markers (Fig. 1), which suggest that, for retinoblastoma stem-like cells isolation, the process of single cell cloning is necessary.

The expression of cell surface markers in normal or stem cells largely depends on the cellular environment and the types of cells [22]. For example, CD24 is upregulated in several types of cancer stem

cells, such as colorectal cancer stem cells, while, downregulation of CD24 is one of the characteristics of breast cancer stem cells [23, 24]. Furthermore, the regulation of stem cell markers may be a part of cellular response to environment, such as drug treatment [25]. Thus, it is important to identify the drivers in stemness maintenance of cancer stem cells. In this study, we found that SOX2 is one of the important drivers, which positively correlates with other stem cell markers in retinoblastoma tissues and derives sphere formation as well as the expression of other stem cell markers in isolated retinoblastoma stem-like cells (Fig. 2–4). Thus, SOX2 could be served as an important therapeutic marker for eradicating retinoblastoma stem-like cells.

SOX2 is well-known as transcription factor, while, development of small molecules targeting transcription factor is challenging [26]. Thus, the feasible strategy targeting SOX2 is to suppress the main downstream signaling of SOX2. Hippo/YAP signaling controls cell fate and tissue size in mammalian, which employ transcription co-activator YAP and its analogue TAZ (encoded by WWTR1). Aberrant activation of YAP frequently happens in both stem and differentiated cancer cells and drives the stemness in several types of cancer stem cells, that several YAP inhibitors have been developed for anti-cancer use. The results of this study demonstrate that SOX2 transcriptionally activates YAP and WWTR1, activates Hippo/YAP signaling, thereby supporting the stemness of retinoblastoma stem-like cells (Fig. 5–7 and 8). The finding also suggests that inhibition of Hippo/YAP signaling is an effective strategy for suppressing SOX2 in retinoblastoma stem-like cells.

In summary, in this study, a method for establishment of retinoblastoma stem-like cells was developed and an important mechanism for stemness maintenance of retinoblastoma stem-like cells, SOX2-drived activation of Hippo/YAP signaling pathway, was revealed. The finding here suggest that YAP suppression should be effective therapeutic strategy for human retinoblastoma cancer caused by aberrant upregulation of SOX2.

Abbreviations

Cancer stem cells, CSCs; American Type Culture Collection, ATCC; Fetal bovine serum, FBS; Recombinant human epidermal growth factor, rhEGF; Recombinant human basic fibroblast growth factor, rh-bFGF; Recombinant human leukemia inhibitory factor, rh-LIF; Real-time quantitative reverse-transcription PCR, qRT-PCR; Chromatin immunoprecipitation quantitative PCR, ChIP-PCR.

Declarations

Ethics approval and consent to participate

The study was approved by the local Ethic Committee.

Consent for publication

All the authors have participated, and have approved the submitted manuscript.

Availability of data and materials

All information associated with the study is available in online version or from the corresponding authors.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Q.L, S.W, Y.S, Y.D, M.Y, H.H performed bioinformatic analysis. T.L performed cell- and molecular-based experiments. N.Z performed experiments associated with xenograft model and participated all the experiments. L.Z participated data analysis and result interpretation. N.Z and T.L supervised the project and wrote the manuscript.

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Figures

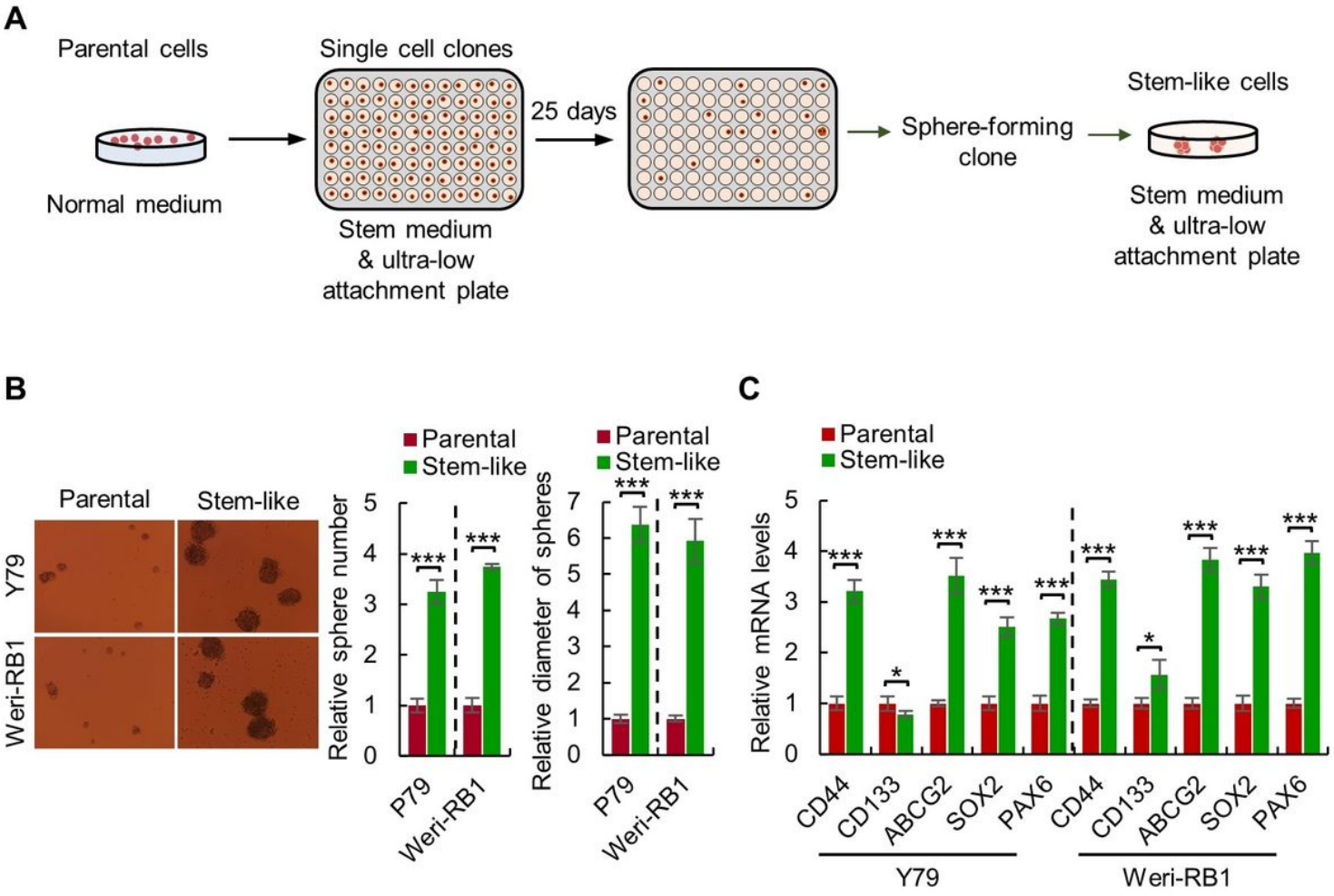


Figure 1

Characterization of retinoblastoma stem-like cells. (A) Isolation of retinoblastoma stem-like cells. Single cells derived from cultured retinoblastoma cell lines were seeded into ultra-low attachment 96 well plate and cultured with serum-free culture medium. The cells with ability of forming spheres were isolated and further cultured as retinoblastoma stem-like cells. (B) Isolated retinoblastoma stem-like cells exhibited enhanced sphere-forming capacities. The sphere-forming capacities of parental cells and isolated stem-like cells were determined by sphere-forming assay. Student t-test (n = 3). (C) The expression of stem cell markers in isolated retinoblastoma stem-like cells. The expression of indicated stem cell markers was determined by qRT-PCR. Student t-test (n = 3). *α < 0.05, **α < 0.01, ***α < 0.001.

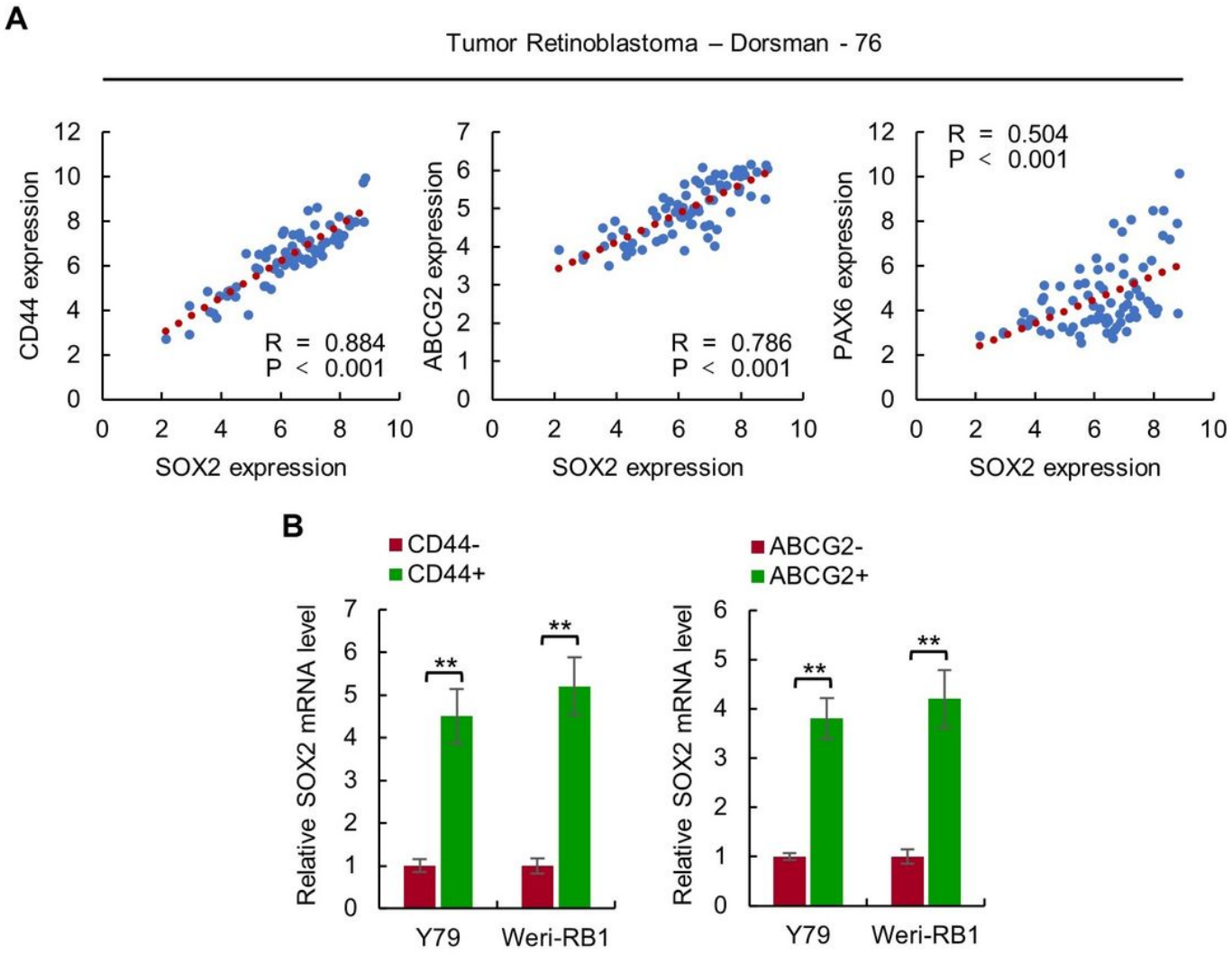
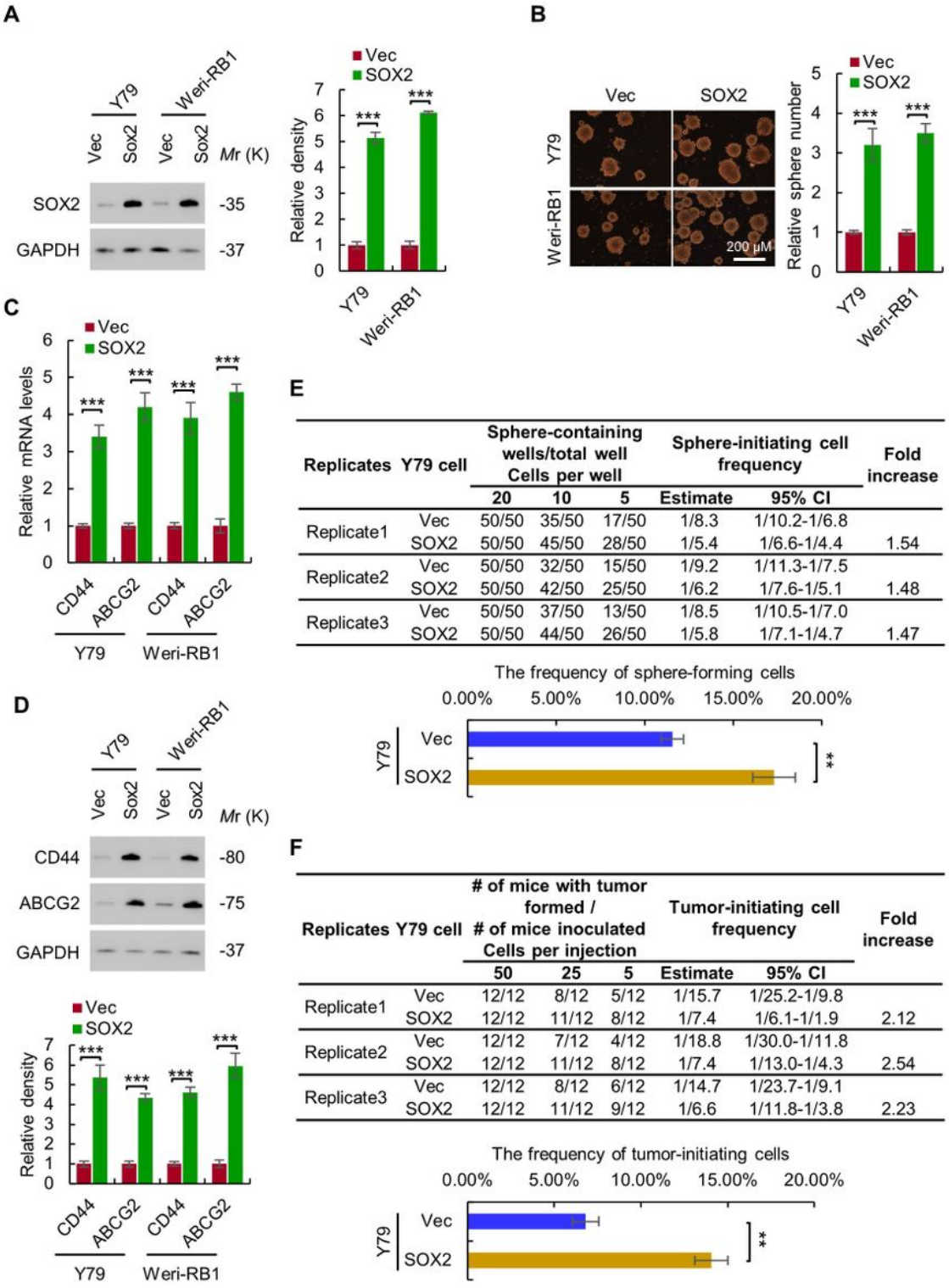


Figure 2

SOX2 is associated with retinoblastoma stemness. (A) The expression of SOX2 is positively correlated with retinoblastoma stem cell markers in tumor samples of retinoblastoma patients. The expression data of SOX2, CD44, ABCG2 and PAX6 in retinoblastoma patients were downloaded. The correlation between SOX2 and CD44, ABCG2 and PAX6 was analyzed by Spearman analysis. (B) The SOX2 expression is upregulated in CD44+ and ABCG+ retinoblastoma cells. The CD44+ and ABCG+ cells were isolated by magnetic beads. The mRNA levels of SOX2 in these cells were analyzed by qRT-PCR. Student t-test (n = 3). *□ < 0.05, **□ < 0.01, ***□ < 0.001.



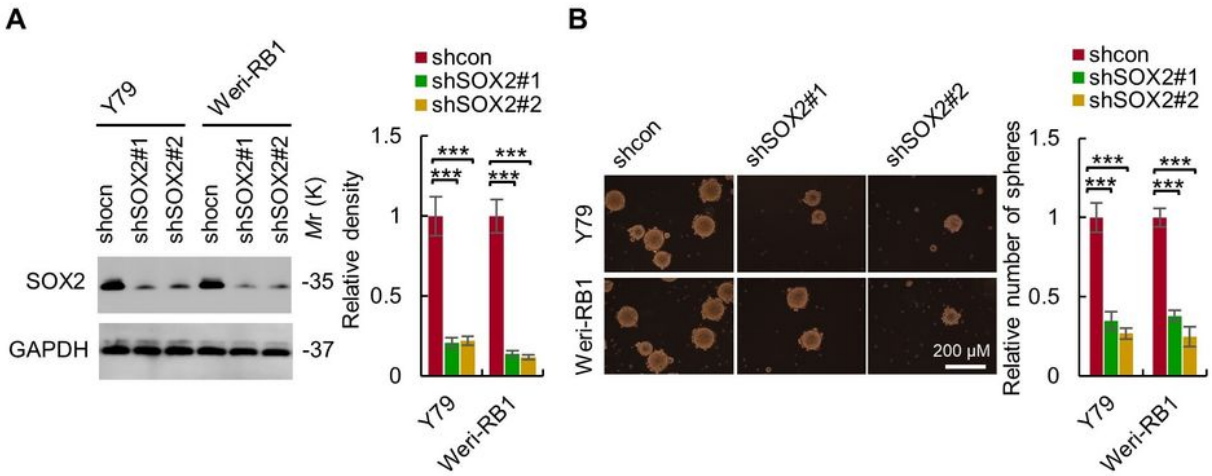
Replicates	Y79 cell	Sphere-containing wells/total well Cells per well			Sphere-initiating cell frequency		Fold increase
		20	10	5	Estimate	95% CI	
Replicate1	Vec	50/50	35/50	17/50	1/8.3	1/10.2-1/6.8	
	SOX2	50/50	45/50	28/50	1/5.4	1/6.6-1/4.4	1.54
Replicate2	Vec	50/50	32/50	15/50	1/9.2	1/11.3-1/7.5	
	SOX2	50/50	42/50	25/50	1/6.2	1/7.6-1/5.1	1.48
Replicate3	Vec	50/50	37/50	13/50	1/8.5	1/10.5-1/7.0	
	SOX2	50/50	44/50	26/50	1/5.8	1/7.1-1/4.7	1.47

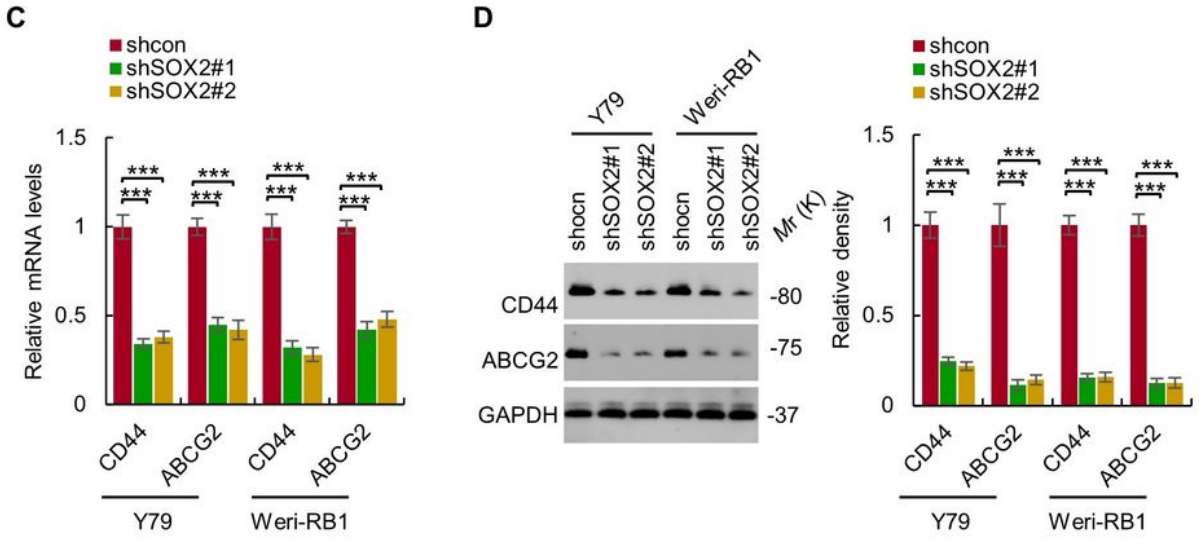
Replicates	Y79 cell	# of mice with tumor formed / # of mice inoculated Cells per injection			Tumor-initiating cell frequency		Fold increase
		50	25	5	Estimate	95% CI	
Replicate1	Vec	12/12	8/12	5/12	1/15.7	1/25.2-1/9.8	
	SOX2	12/12	11/12	8/12	1/7.4	1/6.1-1/1.9	2.12
Replicate2	Vec	12/12	7/12	4/12	1/18.8	1/30.0-1/11.8	
	SOX2	12/12	11/12	8/12	1/7.4	1/13.0-1/4.3	2.54
Replicate3	Vec	12/12	8/12	6/12	1/14.7	1/23.7-1/9.1	
	SOX2	12/12	11/12	9/12	1/6.6	1/11.8-1/3.8	2.23

Figure 3

Ectopic expression of SOX2 enhances the stemness of retinoblastoma stem-like cells. (A) Characterization of SOX2-overexpressing cells. The expression of SOX2 in SOX2-overexpressing and control cells was determined by western blot. (B) The sphere-forming capacities of indicated retinoblastoma stem-like cells were examined by sphere formation assay. Student t-test (n = 3). (C) The mRNA levels of CD44 and ABCG2 in indicated retinoblastoma stem-like cells were examined by qRT-PCR.

Student t-test (n = 3). (D) The protein levels of CD44 and ABCG2 in indicated retinoblastoma stem-like cells were examined by western blot. (E) The sphere-forming cell frequency of indicated retinoblastoma stem-like cells were examined by limiting dilution assay. Student t-test (n = 3 groups). (F) The tumor-initiating cell frequency of indicated retinoblastoma stem-like cells were examined by xenograft assay. Student t-test (n = 3 groups). Student t-test (n = 3). *□ < 0.05, **□ < 0.01, ***□ < 0.001.





E

Y79	Sphere-containing wells/total well			Sphere-forming cell frequency		Fold decrease
	Cells per well			Estimate	95% confidence interval	
	20	10	5			
shocn	50/50	38/50	19/50	1/7.6	1/9.3-1/6.2	
shSOX2#1	49/50	25/50	10/50	1/11.9	1/14.7-1/9.6	1.57
shSOX2#2	48/50	23/50	8/50	1/13.1	1/16.3-1/10.6	1.72

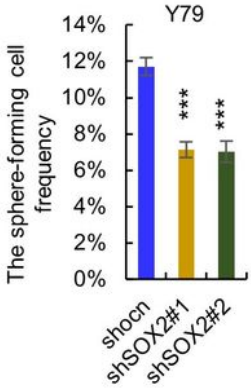


Figure 4

Knockdown of SOX2 attenuates the stemness of retinoblastoma stem-like cells. (A) Characterization of SOX2-knockdown cells. The expression of SOX2 in SOX2-knockdown and control cells was determined by western blot. (B) The sphere-forming capacities of indicated retinoblastoma stem-like cells were examined by sphere formation assay. One-way ANOVA (n = 3). (C) The mRNA levels of CD44 and ABCG2 in indicated retinoblastoma stem-like cells were examined by qRT-PCR. One-way ANOVA (n = 3). (D) The protein levels of CD44 and ABCG2 in indicated retinoblastoma stem-like cells were examined by western blot. (E) The sphere-forming cell frequency of indicated retinoblastoma stem-like cells were examined by limiting dilution assay. One-way ANOVA (n = 3 groups). *P < 0.05, **P < 0.01, ***P < 0.001.

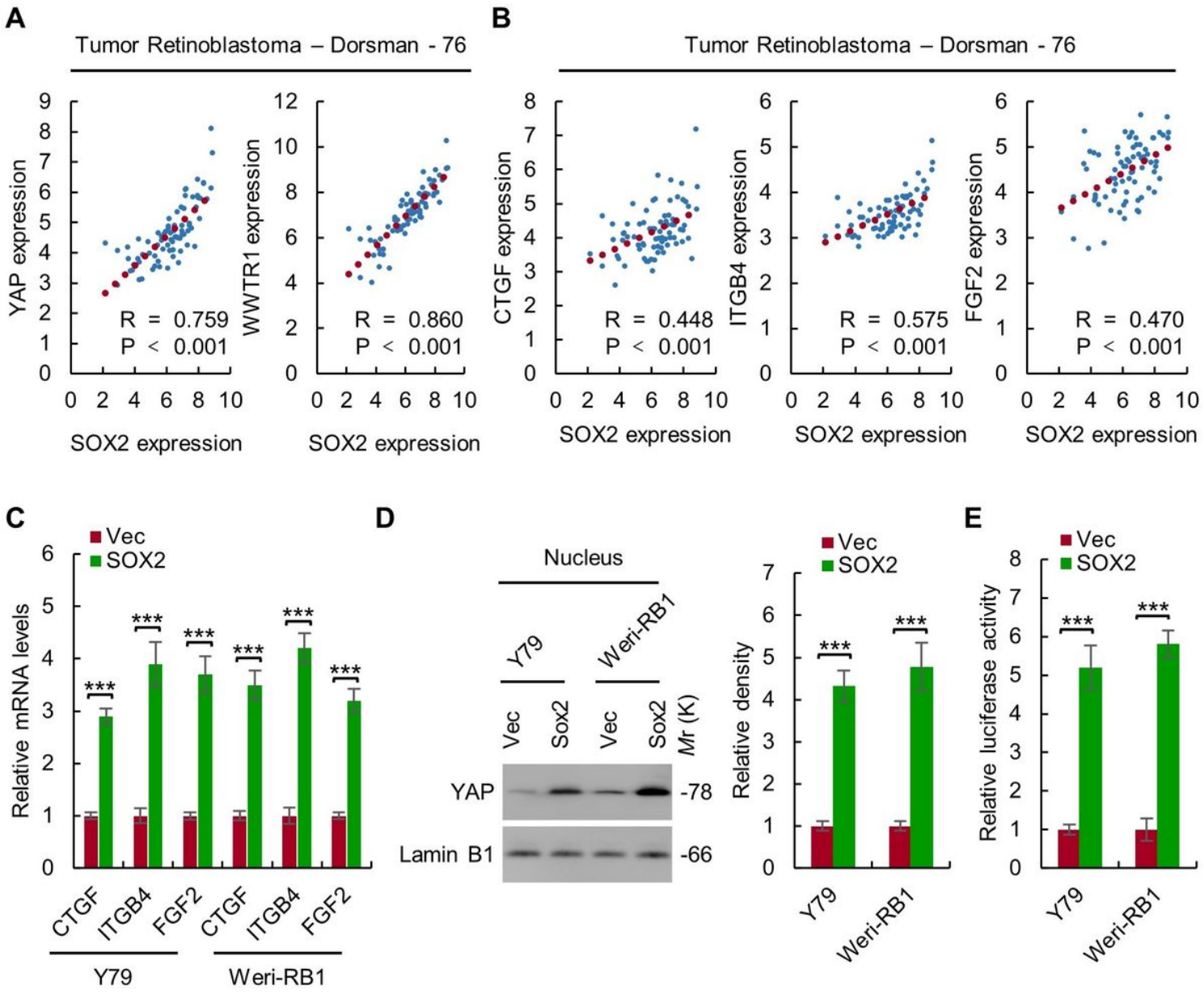


Figure 5

SOX2 activates Hippo/YAP signaling in retinoblastoma stem-like cells. (A,B) The expression of SOX2 is positively correlated with YAP1, WWTR1 (A) and CTGF, ITGB4, FGF2 (B) in tumor tissues of

retinoblastoma patients. The expression data of SOX2, YAP1, WWTR1, CTGF ITGB4 and FGF2 in retinoblastoma patients were downloaded. The correlation between SOX2 and YAP1, WWTR1, CTGF ITGB4 and FGF2 was analyzed by Spearman analysis. (C) The mRNA levels of CTGF, ITGB4, FGF2 in indicated cells were determined by qRT-PCR. Student t-test (n = 3). (D) The protein levels of YAP in nucleus of indicated cells were determined by western blot assay. (E) The transcriptional activity of YAP in indicated cells was determined by luciferase reporter assay. Student t-test (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001.

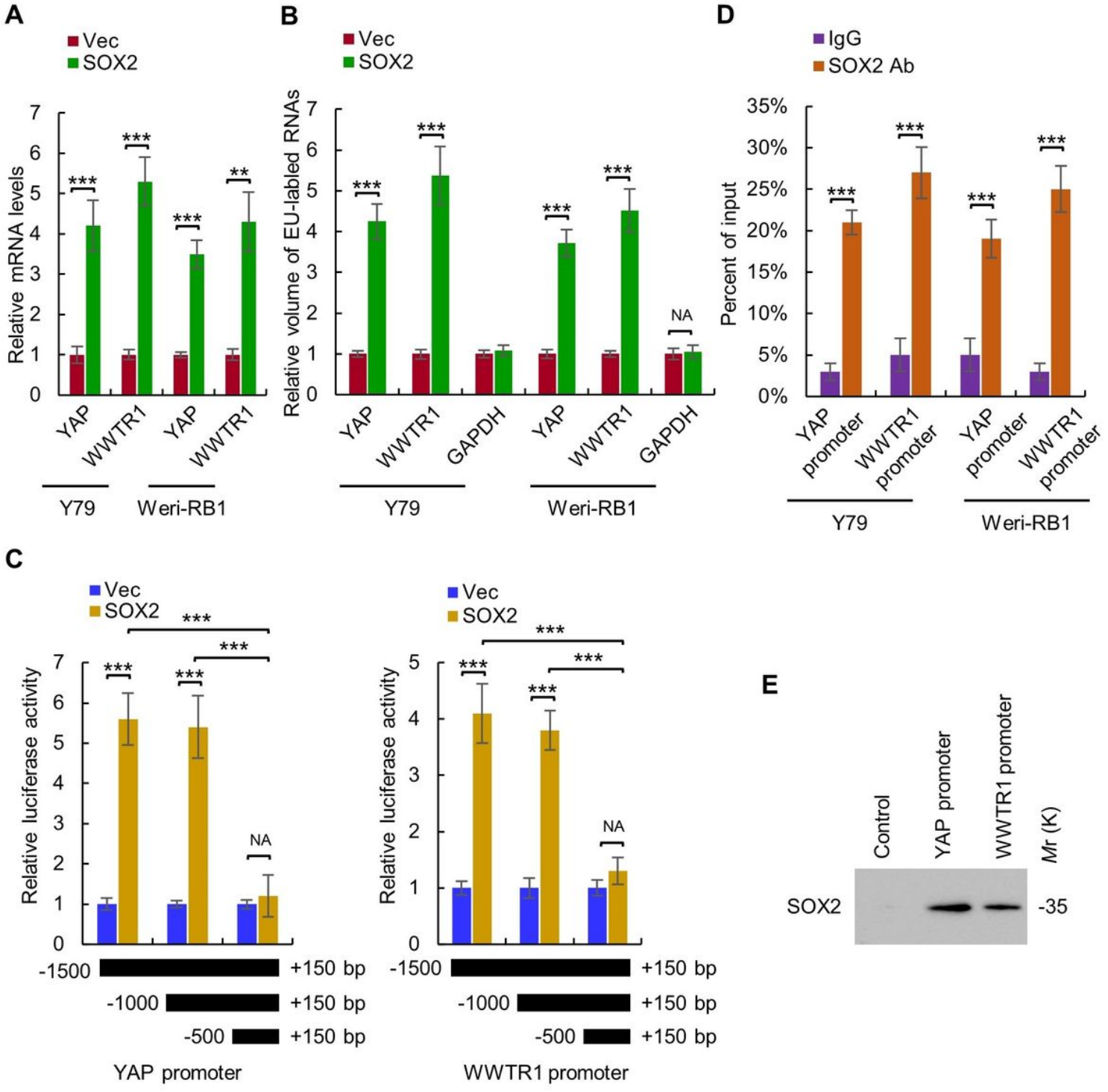


Figure 6

SOX2 transcriptionally activates YAP and WWTR1. (A) The mRNA levels of YAP and WWTR1 in indicated cells were determined by qRT-PCR assay. Student t-test (n = 3). (B) The transcription of YAP, WWTR1 and GAPDH in indicated cells was determined by nuclear run-on assay. (C) The transcriptional activities of different fragments of YAP promoter and WWTR1 promoter in indicated cells were determined by luciferase reporter assay. Student t-test (n = 3). (D) The direct bindings between SOX2 and YAP promoter and WWTR1 promoter were determined by ChIP-PCR assay. Student t-test (n = 3). (E) The direct binding between SOX2 and YAP promoter and WWTR1 promoter were determined by DNA pull-down assay. *□ < 0.05, **□ < 0.01, ***□ < 0.001.

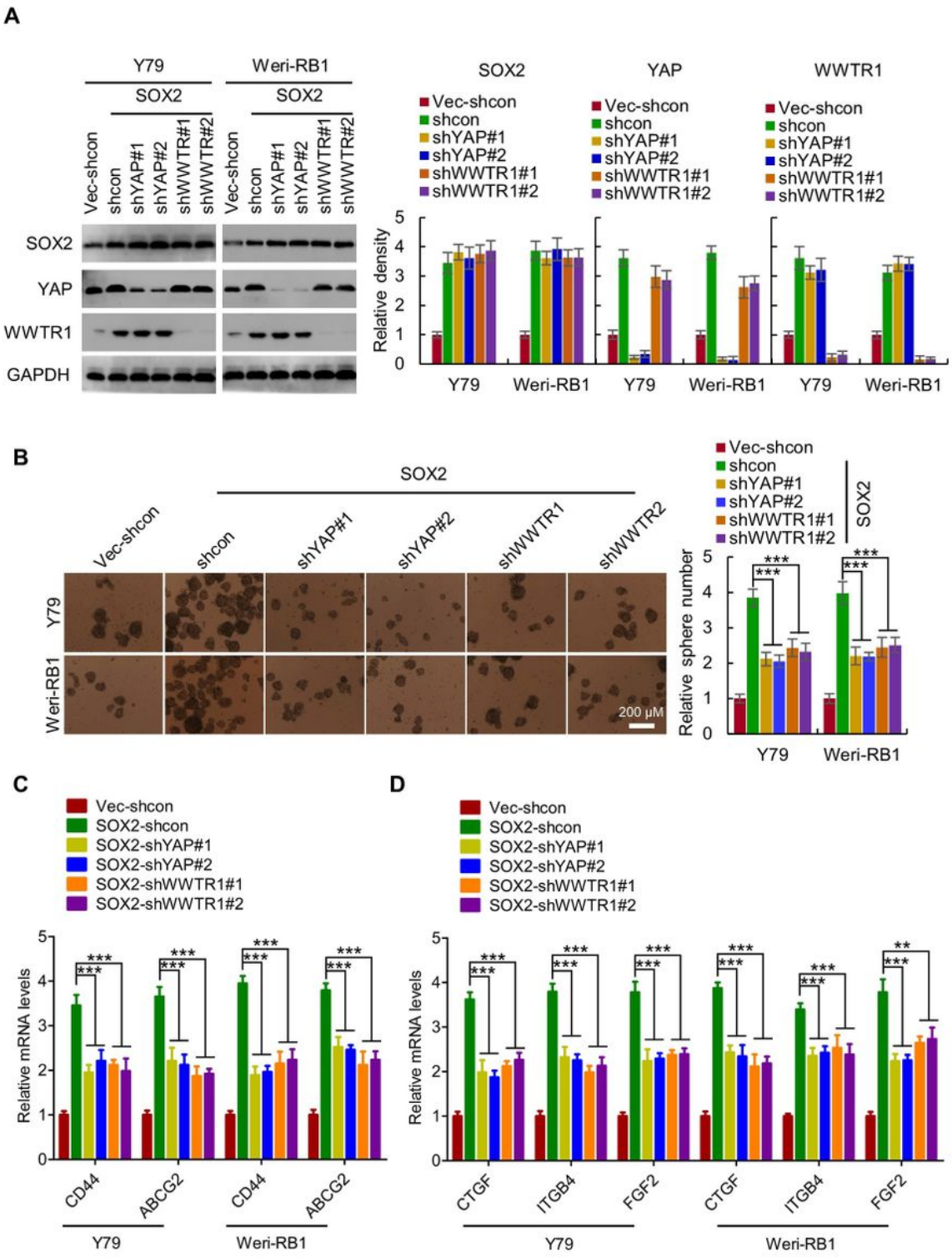


Figure 7

WWTR1 and YAP is necessary for SOX2 promoting the stemness of retinoblastoma stem-like cells. (A) Characterization of SOX2-overexpressing YAP-knockdown or WWTR1-knocdown retinoblastoma stem-like cells. The protein levels of SOX2, YAP and WWTR1 in indicated cells were determined by western blot. (B) The sphere-forming capacities of indicated cells were determined by sphere formation assay. One-way

ANOVA (n = 3). (C,D) The mRNA levels of CD44, ABCG2 (C), CTGF, ITGB4 and FGF2 (D) in indicated cells were determined by qRT-PCR. One-way ANOVA (n = 3). *□ < 0.05, **□ < 0.01, ***□ < 0.001.

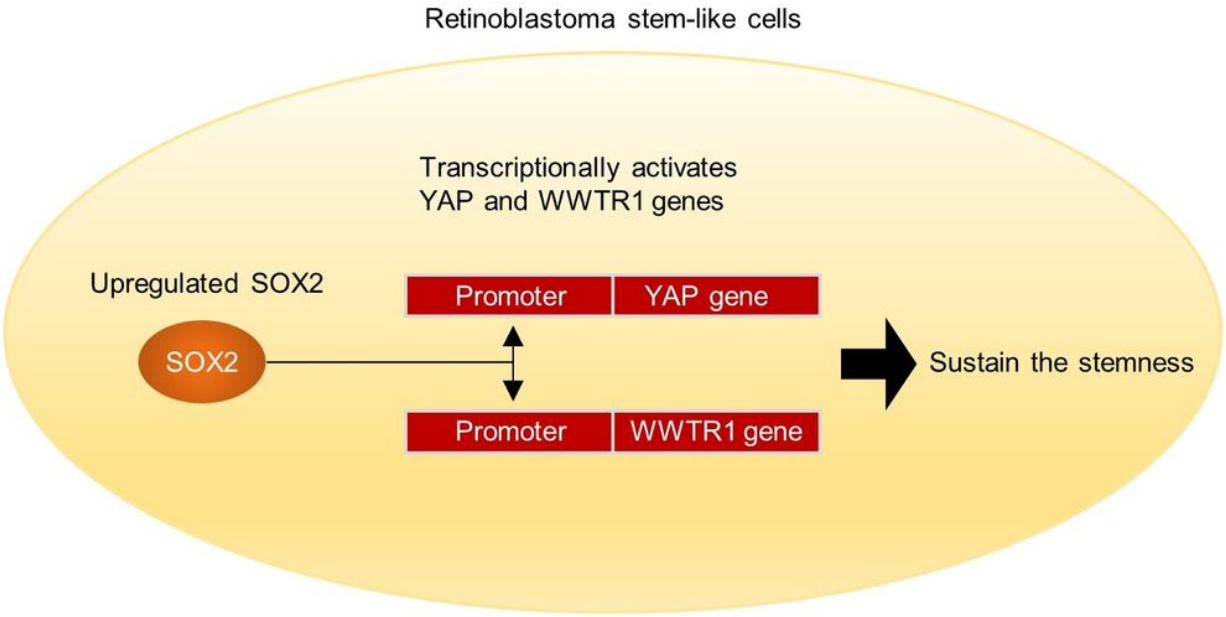


Figure 8

Model for SOX2-drived retinoblastoma stemness maintenance. Upregulated SOX2 in retinoblastoma stem cells directly binds the promoters of YAP and WWTR1, transcriptionally activates YAP and WWTR1, promotes Hippo/YAP signaling, thereby promoting retinoblastoma stemness maintenance.

Supplementary Files

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