AMERICAN SOCIETY of
GENE & CELL
THERAPY

circGprc5a Promoted Bladder Oncogenesis and Metastasis through Gprc5a-Targeting Peptide

Chaohui Gu,[1,3] Naichun Zhou,[1,3] Zhiyu Wang,[1] Guanru Li,[1] Yipping Kou,[1] Shunli Yu,[1] Yongjie Feng,[1] Long Chen,[1] Jinjian Yang,[1] and Fengyan Tian[2]

[1]Department of Urology and Henan Institute of Urology, Zhengzhou Key Laboratory for Molecular Biology of Urological Tumor Research, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, P.R. China; [2]Department of Pediatrics, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, P.R. China

Bladder cancer is a serious cancer in the world, especially in advanced countries. Bladder cancer stem cells (CSCs) drive bladder tumorigenesis and metastasis. Circular RNAs (circRNAs) are involved in many biological processes, but their roles in bladder oncogenesis and bladder CSCs are unclear. Here, we identified that circGprc5a is upregulated in bladder tumors and CSCs. circGpr5a knockdown impairs the self-renewal and metastasis of bladder CSCs, and its overexpression exerts an opposite role. circGpr5a has peptide-coding potential and functions through a peptide-dependent manner. circGprc5a-peptide binds to Gprc5a, a surface protein highly expressed in bladder CSCs. *Gprc5a* knockout inhibits the bladder CSC self-renewal and metastasis. circGprc5a-peptide-Gprc5a can be utilized to target bladder cancer and bladder CSCs.

INTRODUCTION

As one of most serious cancers in the world, bladder cancer leads to hundreds of thousands of death each year.[1] Several kinds of cells exist in the bladder tumor bulk, including bladder cancer stem cells (CSCs).[2] Bladder CSCs induce tumorigenesis vigorously, and non-CSCs cannot.[2] With self-renewal and differentiation capacities, bladder CSCs are the origin of bladder oncogenesis and metastasis. There are several surface markers identified in bladder CSCs, and CD44 is one of the most widely accepted markers.[3] Considering the characteristics of bladder CSCs, scientists have developed several assays to examine bladder CSCs, including side population, sphere formation, tumor propagation, tumor-initiating assay, and trans-well-invasion assay.[4] In fetal bovine serum (FBS)-free medium, bladder CSCs can escape anoikis and survive to form stem-like onco-spheres.[5] Highly expressing ABCG2 and other pumps, CSCs are resistant to drug treatment.[6] Despite of the importance of bladder CSCs in bladder tumorigenesis and metastasis, the biology of bladder CSCs is unclear.

The activity of CSCs is precisely regulated, and several signaling pathways are involved in CSC self-renewal, including Wnt/β-catenin, Notch, Hedgehog, protein kinase C (PKC), and Hippo/Yap signaling pathways.[7–10] Some key transcription factors are also modulators for CSC fate determination and self-renewal.[11,12] Recently, increasing long non-coding RNAs (lncRNAs) are involved in CSC self-renewal.[13,14] LncRNAs exert their roles by modulating the stability or activity of partner proteins to regulate the activity of CSC-related pathways.[15] G-protein-coupled receptor (GPCR) signaling is one of the most important pathways in signal transduction, however, its role in bladder CSCs is largely unknown.

Circular RNAs (circRNAs) are a kind of circular RNA molecule and exist in many species. circRNAs were identified a long time ago, and their roles were discovered recently.[16] As a new kind of functional RNA, circRNAs are involved in many physiological and pathological processes, including stemness regulation and tumorigenesis[17]. As oncogenic factors or tumor repressors,[18] circRNAs exert their roles in tumor propagation, metastasis,[19] and radioresistance.[20] circRNAs function via various mechanisms. They regulate the concentration of microRNA (miRNA),[16,21] modulate the stability or activity of their partners, bind to the target gene promoter, recruit transcription-associated factors,[22] or modulate the expression of target genes. Recently, increasing works revealed the peptide-coding potential of circRNAs, and some circRNAs exert their through the peptide.[23–25] Although increasing roles of circRNAs were identified, their roles in CSCs are unknown.

Here, we discovered that circGprc5a was upregulated in bladder tumors and bladder CSCs and positively regulated the activity of bladder CSCs. circGprc5a had peptide coding potential and functioned through the peptide. circGprc5a-peptide-Gprc5a could also be targeted for bladder tumor and bladder CSC elimination. All together, our work identified a functional circRNA in bladder tumorigenesis and bladder CSCs, adding a novel layer for bladder CSC regulation and a novel function of circRNA.
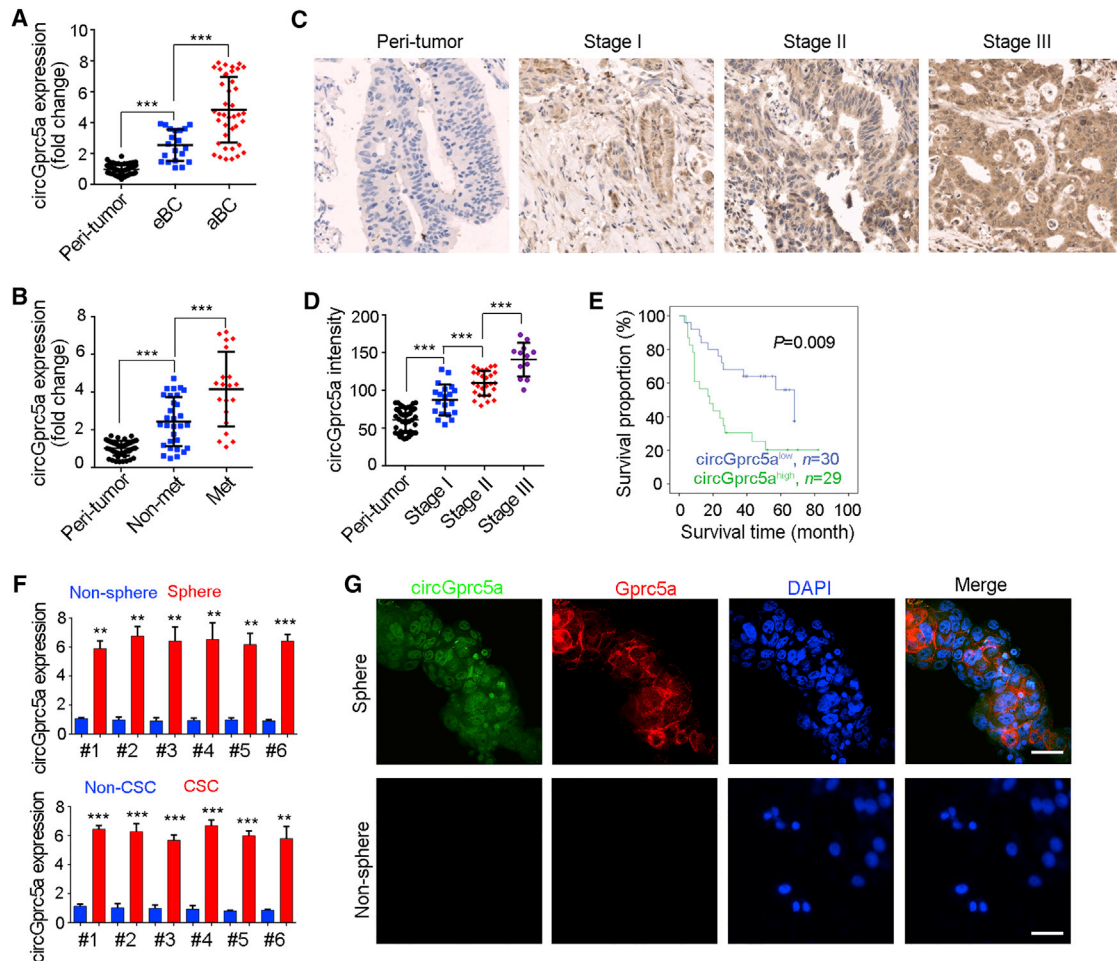
Figure 1. Upregulation of circGprc5a in Bladder Tumor and CSCs

(A) Real-time PCR results of circGprc5a. 60 peri-tumor samples, 20 early bladder cancer samples, 40 advanced bladder cancer samples were used for real-time PCR. All expression levels were normalized to the average expression levels of peri-tumor samples. (B) circGprc5a expression in metastatic tumors. 50 peri-tumor, 30 non-metastatic, and 20 metastatic tumors were used for circGprc5a detection. (C and D) Bladder tumor tissue array was used for circGprc5a *in situ* hybridization (ISH). Typical images were shown in (C), and calculated photon intensities were shown in (D). (E) According to circGprc5a intensity, bladder patients were divided into two groups (circGprc5a^{high} and circGprc5a^{low}), and Kaplan-Meier survival analysis was performed using SPSS 20. Samples with circGprc5a intensity > average intensity were grouped into circGprc5a^{high}, and other samples were grouped into circGprc5a^{low}. (F) circGprc5a expression in bladder CSCs and oncospheres. In the upper panel, sphere formation assay was performed, and spheres and non-spheres were collected. For bladder CSCs, CD44$^+$ cells were stained and enriched by FACS. (G) Bladder oncospheres and non-spheres were collected for circGprc5a staining, counterstained with Gprc5a and DAPI, and samples were observed by confocal microscopy. Scale bars, 20 μm. **p < 0.01, ***p < 0.01 by unpaired one-tailed Student's t test.

RESULTS

circGprc5a Was Upregulated in Bladder Cancer and Bladder CSCs

Increasing incidence and mortality of bladder cancer occur in many countries, and the biological regulation of bladder cancer is largely unknown. circRNAs are modulators in many biological processes. Among the various circRNAs we examined, here we identified a circRNA (hsa_circ_02838, named circGprc5a because its peptide combined with Gprc5a) was upregulated in bladder tumors (Figures 1A and 1B). Moreover, the expression of circGprc5a was also related to clinical severity and metastasis of bladder cancer. Most high expression of circGprc5a was detected in advanced

bladder tumors and in metastatic tumors (Figures 1A and 1B). To deeply examine the expression levels of circGprc5a, bladder tumor tissue array was used for circGprc5a *in situ* hybridization (ISH), and the results also confirmed the high expression of circGprc5a in bladder cancer and serious bladder cancer (Figures 1C and 1D). Interesting, circGprc5a expression was also related to clinical outcome. circGprc5a highly expressed tumors showed bad prognosis, and lowly expressed tumors showed good prognosis (Figure 1E).

We then enriched bladder CSCs through surface markers and detected circGprc5a expression, revealing the high expression of
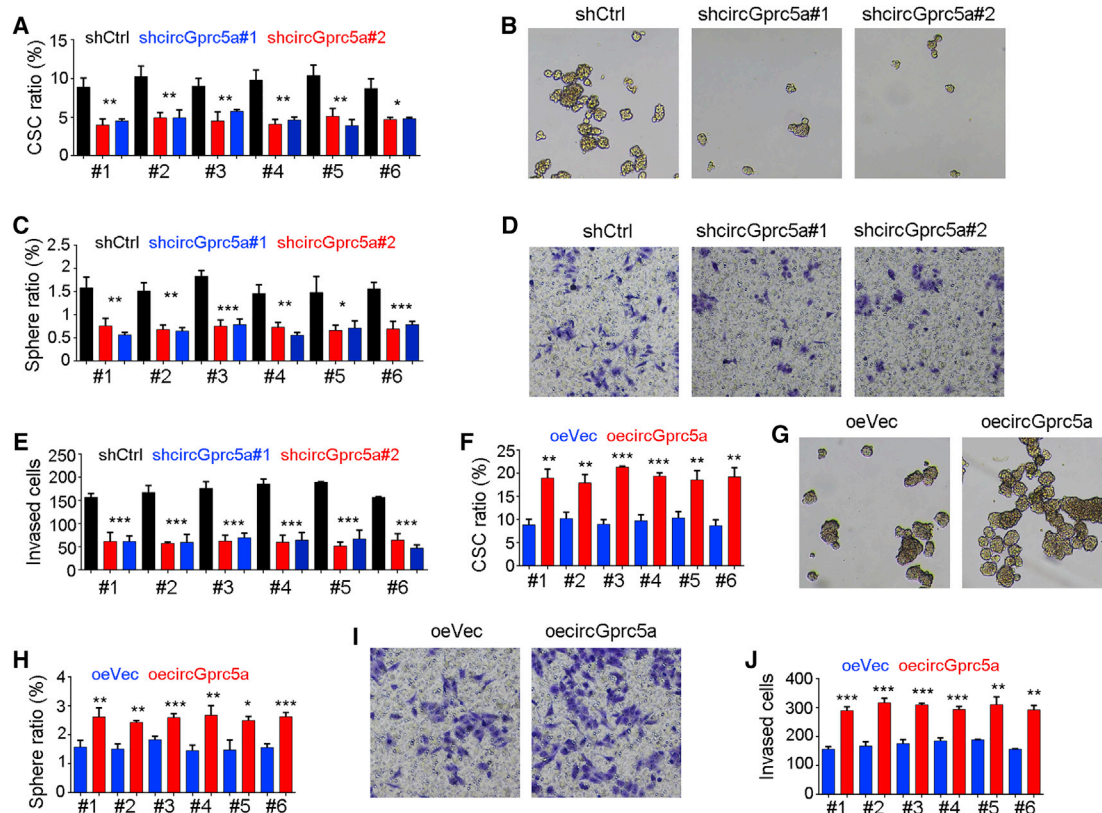
Figure 2. circGprc5a Drove Bladder CSC Self-Renewal

(A) Bladder CSC ratios of circGprc5a-silenced cells. circGprc5a-silenced cells were generated through lentivirus, and bladder CSCs were detected by CD44 staining and FACS detection. (B and C) Spheres (B) and sphere-initiating ratios (C) of circGprc5a-silenced cells. circGprc5a knockdown cells were used for sphere-formation assay. Two weeks later, sphere photos were taken, and sphere formation ratios were calculated. (D and E) Invasive cells (D) and cell numbers (E) of circGprc5a knockdown cells. circGprc5a-silenced cells were utilized for transwell invasion assay. 36 hr later, invasive cells were visualized by crystal violet staining (D), and cell numbers were counted (E). (F) circGprc5a-overexpressing cells were established and bladder CSCs were detected. oe, overexpression. (G and H) circGprc5a-overexpressing spheres (G) and sphere-formation ratios (H). circGprc5a-overexpressing cells were incubated into sphere-formation medium, and spheres were observed 2 weeks later (G). Sphere-initiating ratios were calculated and shown in (H). (I and J) Transwell assay of circGprc5a-overexpressing cells. circGprc5a-overexpressing cells were used for transwell-invasion assay. 36 hr later, invasive cells were observed (I), and cell numbers were counted (J). *$p < 0.05$, **$p < 0.01$, ***$p < 0.01$ by unpaired one-tailed Student's t test.

circGprc5a in bladder CSCs (Figure 1F). Oncospheres were also collected, and circGprc5a was detected by real-time PCR and fluorescence in situ hybridization (FISH) assay. The results also showed a high expression of circGprc5a in the oncosphere (Figure 1G). All together, increased circGprc5a expression was observed in bladder tumors and bladder CSCs.

circGprc5a Drove Bladder CSC Self-Renewal

To investigate the role of circGprc5a in bladder CSCs, we generated two circGprc5a knockdown cells and examined the ratios of bladder CSCs through fluorescence-activated cell sorting (FACS). Impaired CSC ratios were detected upon circGprc5a knockdown, indicating the essential role of circGprc5a in CSC maintenance (Figure 2A). Sphere-formation assay was also performed, and impaired sphere-formation capacity was observed upon circGprc5a knockdown, revealing the critical role of circGprc5a in sphere formation (Figures 2B and 2C). Similarly, circGprc5a

depletion also inhibited the metastasis of bladder CSCs (Figures 2D and 2E).

We then generated circGprc5a-overexpressing cells with lentivirus infection and examined bladder CSCs through FACS. On the contrary, with circGprc5a knockdown cells, circGprc5a-overexpressing cells showed higher ratios of bladder CSCs, confirming the role of circGprc5a in bladder CSC maintenance (Figure 2F). circGprc5a-overexpressing cells were also utilized to sphere formation and transwell invasion assays, and the results proved the critical role of circGprc5a in bladder self-renewal and invasion capacity (Figures 2G–2J). All together, circGprc5a drove the self-renewal and metastasis of bladder CSCs.

circGprc5a Encoded Peptide to Drive CSC Self-Renewal and Metastasis

Increasing evidences proved the peptide-coding potential of circRNAs, and here we also detected the coding potential or
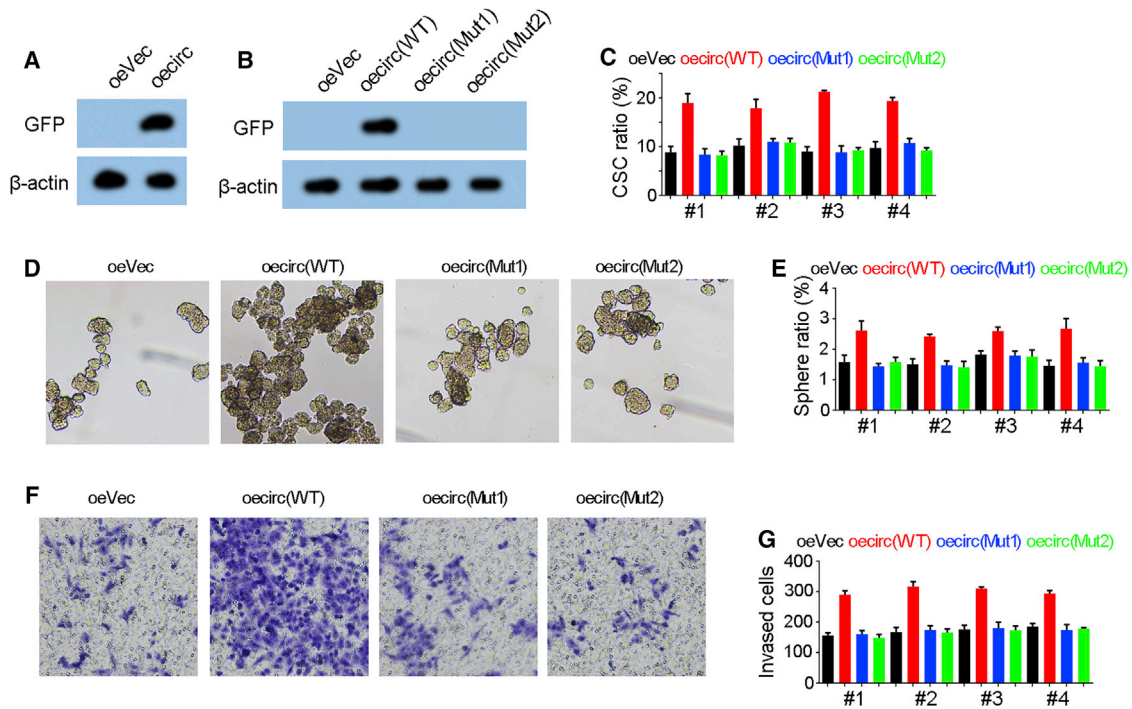
Figure 3. circGprc5a Produced Peptide and Acted through Peptide

(A) The coding potential of circGprc5a was detected by western blot. β-actin was a loading control. (B) Two mutations with no peptide coding potential were constructed, and coding potential was detected by western blot. (C) Bladder CSCs in WT and mutant circGprc5a. circGprc5a mutants were overexpressed in bladder tumor cells, and CSCs were examined by FACS with CD44 antibody. (D and E) Sphere-formation capacity of circGprc5a mutant cells. circGprc5a mutant and control cells were used for sphere-formation assay. Two weeks later, spheres were observed (D), and sphere formation ratios were shown (E). (F and G) Transwell assay of circGprc5a mutant cells. circGprc5a mutant cells were used for transwell assay. 36 hr later, invasive cells were observed (F), and cell numbers were counted (G).

circGprc5a. circGprc5a showed strong coding potential (Figure 3A), and the peptide sequence was identified as FDTKPMNLCGR. To examine the potential role of circGprc5a-peptide, we generated two circGprc5a mutations and found impaired coding capacities: one is to change the first amino acid "F" to stop codon TAG, and the other is to change the second amino acid "D" to stop codon TAG (Figure 3B).

We utilized circGprc5a mutations to examine the role of circGprc5a in bladder CSC maintenance, self-renewal, and metastasis. As shown in Figure 3C, wild-type (WT) circGprc5a overexpression led to an increased CSC ratios, but mutant circGprc5a had no influence on bladder CSC ratios, indicating the critical role of circGprc5a in bladder CSC maintenance. Sphere formation assay showed impaired role of mutate circGprc5a, confirming the critical role of circGprc5a-peptide in sphere formation (Figures 3D and 3E). Similarly, the essential role of circGprc5a-peptide in transwell assay was also confirmed (Figures 3F and 3G). Taken together, circGprc5a had peptide-coding potential and exerted its role in a peptide-dependent manner.

circGprc5a-Peptide Bound to Gprc5a

To further explore the mechanism of circGprc5a and its peptide, we performed pull-down assay and identified Gprc5a as a partner of circGprc5a through mass spectrum (Figure 4A). The combination of circGprc5a-peptide and Gprc5a was confirmed by western blot (Figure 4B).

We then explored the expression of Gprc5a in bladder CSCs. As shown in Figures 4C and 4D, Gprc5a was highly expressed in bladder tumors through real-time PCR. Moreover, Gprc5a was most highly expressed in advanced bladder tumors and metastatic bladder tumors, indicating the relation between Gprc5a expression and bladder clinical severity and metastasis (Figures 4C and 4D). The high expression of Gprc5a in bladder cancer was confirmed by immunohistochemistry (IHC) (Figure 4E). We then enriched bladder CSCs and oncospheres and examined Gprc5a expression with western blot. The results showed that Gprc5a was highly expressed in bladder CSCs and bladder oncospheres (Figures 4F and 4G). All together, circGprc5a-peptide bound to Gprc5a that was highly expressed in bladder tumors and bladder CSCs.

Gprc5a Exerted an Essential Role in Bladder CSCs

To detect the role of Gprc5a in bladder CSC self-renewal, we generated *Gprc5a* knockout cells through CRISPR/Cas9 approach[26] (Figure 5A). *Gprc5a* knockout cells showed decreased ratios of bladder CSCs, indicating the essential role of Gprc5a in bladder CSC
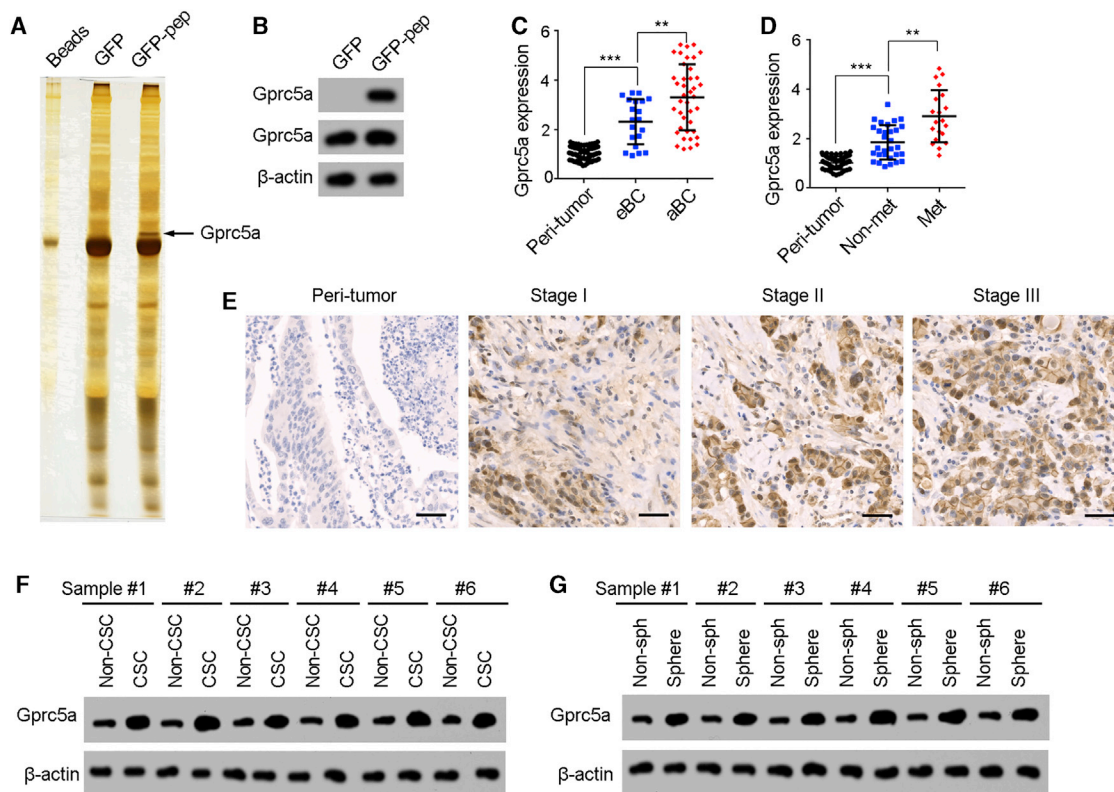
Figure 4. circGprc5a-Peptide Combined with Gprc5a

(A) Silver staining of circGprc5a-peptide pull-down. circGprc5a-peptide and control GFP proteins were incubated with bladder-sphere lysate, and the samples were used for silver staining. The specific band of circGprc5a-peptide sample was identified as Gprc5a with mass spectrum. (B) Western blot for Gprc5a. circGprc5a-peptide pull-down was performed, and the eluate was analyzed by western blot with Gprc5a antibody. (C and D) Real-time PCR results showing the expression of Gprc5a in bladder tumors. For (C), early bladder cancer (eBC) and advanced bladder cancer (aBC) samples were used; for (D), non-metastatic and metastatic samples were used for real-time PCR detection. (E) Gprc5a IHC results in bladder tumors. Bladder tumors were used or Gprc5a IHC detection, and typical images were shown. Scale bars, 50 μm. (F and G) Western blot for Gprc5a expression in bladder CSC (F) and bladder spheres (G). Bladder CSCs (F) and spheres (G) were collected for western blot. Gprc5a expression was analyzed and β-actin was a loading control. **p < 0.01, ***p < 0.01 by unpaired one-tailed Student's t test.

maintenance (Figure 5B). Sphere formation assay was also performed to detect Gprc5a function, and confirmed the essential role of Gprc5a in bladder CSCs (Figures 5C and 5D). Similarly, transwell assay also revealed the importance of Gprc5a in bladder tumor metastasis (Figures 5E and 5F). In conclusion, Gprc5a was required for bladder CSCs.

Considering the importance of circGprc5a-peptide and the combination of Gprc5a and peptide, we examined the role of Gprc5a in circGprc5a function. We overexpressed circGprc5a in Gprc5a knockout cells and found circGprc5a had no effect on bladder CSC ratios, indicating the importance of Gprc5a in circGprc5a function (Figure 5G). Sphere formation assay showed circGprc5a drove the self-renewal of bladder CSCs through Gprc5a-dependent manner (Figures 5H and 5I). The essential role of Gprc5a in circGprc5a function was also confirmed by transwell invasion assay (Figures 5J and 5K). Taken together, circGprc5a was involved in bladder CSC maintenance, self-renewal, and metastasis through Gprc5a-dependent manner.

A Potential Role of circGprc5a-Peptide-Gprc5a Axis in Bladder CSC Targeting

Considering the important role of circGprc5a-peptide-Gprc5a in bladder CSC maintenance, self-renewal, and metastasis, we examined whether circGprc5a-peptide-Gprc5a signaling can be used for bladder CSC targeting. circGprc5a knockdown and Gprc5a knockout cells showed impaired tumor propagation (Figure 6A). The established tumors were obtained for CSC detection and decreased bladder CSCs were found, further confirming the essential role of circGprc5a and Gprc5a in bladder CSC maintenance in vivo (Figure 6B). IHC assay confirmed Gprc5a knockout efficiency in established tumors (Figure 6C). circGprc5a mutant cells also showed impaired tumor propagation and CSC maintenance (Figures 6D and 6E). Taken together, circGprc5a-Gprc5a inhibition led to reduced tumor propagation and bladder CSCs.

We then challenged the role of circGprc5a-peptide-Gprc5a axis in established tumors. circGprc5a and Gprc5a antisense oligos (ASOs) were used to treat 300-mm³ tumors, and reduced tumor volumes
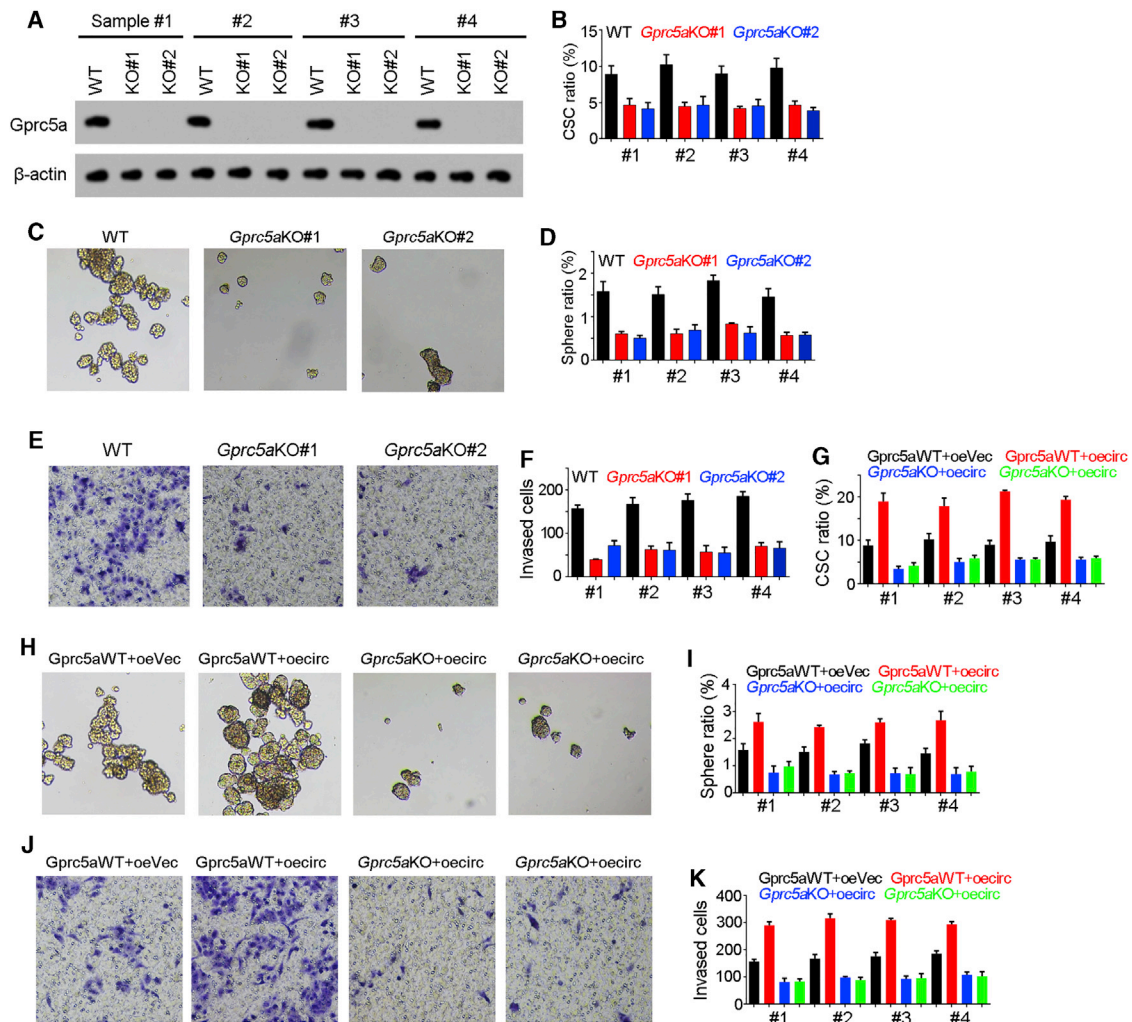
Figure 5. Gprc5a Played an Essential Role in Bladder CSCs

(A) Western blot of Gprc5a knockout cells. *Gprc5a* knockout cells were generated through CRISPR/Cas9 method, and knockout efficiency was detected by real-time PCR. (B) Bladder CSC ratios of *Gprc5a* knockout cells. FACS was performed to detect the ratios of bladder CSCs and the results were shown as mean ± SD. (C and D) Sphere-formation assay of *Gprc5a* knockout cells. *Gprc5a*-deficient cells were incubated in sphere-formation medium for 2 weeks' sphere-formation assay. Spheres were observed (C), and sphere formation assay were calculated (D). (E and F) *Gprc5a* knockout cells were used for transwell invasion assay. Typical invasive cells were shown in (E), and invasive cell numbers were shown in (F). (G) circGprc5a was overexpressed in *Gprc5a* knockout cells, and CSCs were detected by FACS with CD44 antibody. (H and I) circGprc5a-overexpressing *Gprc5a* knockout cells were used for sphere formation. Established spheres were shown in (H), and sphere-initiating ratios were shown in (I). (J and K) Transwell invasion of circGprc5a-overexpressing *Gprc5a* knockout cells. The indicated cells were used for transwell invasion assay. 36 hr later, invasive cells were observed (J) and cell numbers were calculated (K).

were observed upon circGprc5a/Gprc5a inhibition (Figure 6F). All together, circGprc5a-peptide-Gprc5a blockade could be used for bladder targeting and bladder CSC targeting.

DISCUSSION

The self-renewal of bladder CSCs is finely modulated by various regulators. Here, we identified a circRNA in bladder CSCs. circGprc5a was upregulated in bladder tumors and bladder CSCs and played an essential role in bladder CSC self-renewal. Moreover, circGprc5a secreted peptide, which exerted its role in an autocrine manner.

circGprc5a-peptide combined with Gprc5a membrane protein and activated GPCR signaling. circGprc5a-peptide-Gprc5a axis can be used for bladder CSC targeting. Our work identified a functional circRNA in bladder CSC self-renewal, providing a novel regulation of bladder CSCs.

Though increasing signaling pathways are revealed in bladder CSC self-renewal, the role of GPCR signaling in bladder CSCs is unknown. GPCR signaling is the key signaling in signaling transduction and plays a critical role in tumorigenesis.[27] Meanwhile, GPCRs are also
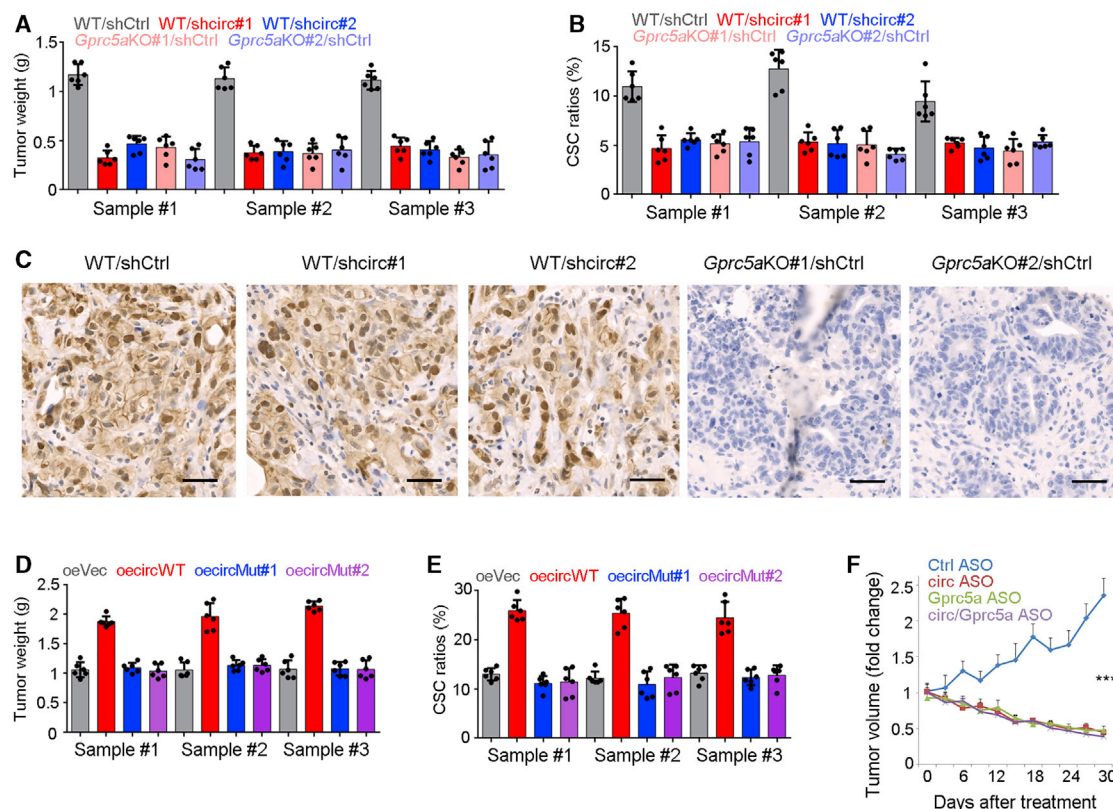
Figure 6. circGprc5a-Peptide-Gprc5a Could Be Used for Bladder CSC Targeting

(A) Tumor weight of the indicated cells. circGprc5a-silenced and *Gprc5a* knockout cells were used for tumor propagation. One month later, tumors were obtained and weight was measured. (B) Bladder CSC ratios in the indicted tumors. The established tumors were obtained and digested into single cells, and bladder CSCs were detected by FACS. (C) 5-μm sections derived from the indicated tumors were used for IHC with Gprc5a antibody, and typical images were shown. Scale bars, 100 μm. (D and E) Tumor weight (D) and bladder CSCs (D) of circGprc5a mutant tumors. WT and mutant circGprc5a-overexpressing cells were used for tumor formation. One month later, tumors were obtained and tumor weight (D) and bladder CSCs (E) were detected as in (A) and (B). (F) Bladder tumors were established, and circGprc5a/Gprc5a ASOs were injected into tumors when tumors were about 300 mm³, and tumor volumes were measured every 3 days.

involved in stemness regulation. Lgr5, a GPCR, was the marker of progenitor cells within various tissues[28–31] and its knockout drive was embryonic lethal,[28] indicating the critical role of Lgr5 in stemness regulation and development. CSCs harbor cancer and stem characteristics simultaneously, and some factors involved in tumorigenesis and stemness are modulators of CSCs.[32] Thus, GPCRs probably drive CSC self-renewal. Here, we identified that Gprc5a was a key factor of bladder CSCs. Taking advantage of sphere-formation assay, transwell-invasion assay, tumor-initiation assay, and *Gprc5a* knockout cells, we validated the essential role of Gprc5a in bladder CSC self-renewal. Our work proved GPCR signaling as a center signaling pathway in bladder CSCs, adding a new layer for CSC regulation and GPCR function.

circRNAs exert their roles through various functional patterns.[33] Some circRNAs act as miRNA sponges and regulate the concentration of miRNAs.[16,34] Some circRNAs interact with RNA polymerase II to regulate transcription.[22] Some circRNAs recruit chromatin-remodeling complexes to the promoter region of target genes.[35] Here,

we found that circGprc5a produced peptide and exerted its role in a peptide-dependent manner. We constructed circGprc5a mutant cells that showed no peptide-coding potential and found an impaired role of circGpr5a mutations, proving the essential role of circGpr5a in circGprc5a function. To further confirm the role of circGprc5a-peptide, we deleted the expression of Gprc5a, the partner of circGprc5a-peptide. *Gprc5a* knockout impaired the self-renewal and metastasis of bladder CSCs. Moreover, circGprc5a overexpression has no influence on bladder CSC self-renewal, confirming the critical role of peptide-Gprc5a in circGprc5a function. The roles of circGprc5a in other biological processes are poorly defined, and it will be interesting to investigate the role of circGprc5a in other processes and whether it functions through peptide-Gprc5c dependent manner.

There are various cells in bladder tumor bulk, and there are large differences between different patients.[36] The heterogeneity of bladder cancer means that most of the transcripts were only highly expressed in a subset of bladder tumors. Here, we identified that circGprc5a was upregulated in most of bladder patients and played essential roles in

CSC self-renewal in all six samples identified. Moreover, circGprc5a-peptide-Gprc5a pathways can be used for bladder CSC targeting. The universal function of circGprc5a-peptide-Gprc5a in bladder cancer and other cancers will be investigated further. All together, our work defined the role of circRNAs and GPCRs in the self-renewal of bladder CSCs.

MATERIALS AND METHODS

Samples and Cell Lines
Primary bladder cells were obtained from The First Affiliated Hospital of Zhengzhou University with informed consent. Primary bladder samples were from the Department of the Urology and Henan Institute of Urology, Zhengzhou Key Laboratory for Molecular Biology of Urological Tumor Research, The First Affiliated Hospital of Zhengzhou University with informed consent. All experiments involving mice were approved by the First Affiliated Hospital of Zhengzhou University. The bladder tumors were digested into single cells for bladder CSC isolation or enrichment and functional detection. For CRISPR/Cas9 lentivirus and PSiCoR lentivirus, lentivirus were packaged in 293T cells, which were maintained in DMEM medium (containing 10% FBS, $1\times$ penicillin, and $1\times$ streptomycin).

circGprc5a Knockdown
For circGprc5a knockdown, the sequences of the circGprc5a junction were used for small hairpin RNA (shRNA) design, and two shRNAs were collected. shRNA was cloned into PSiCoR vector, and transfected into 293T cells for lentivirus package. The purified lentivirus was used for bladder cancer cell infection, and the infected cells were encircled by FACS assay. circGprc5a knockdown efficiency was examined by real-time PCR and then used for functional detection.

Gprc5a Knockout
For *Gprc5a* knockout, Gprc5a small guide RNAs (sgRNAs) were designed according to an online tool (http://crispr.mit.edu/), purchased from Sangon Company and cloned into lentiCRISPRv2 plasmid. CRRISPR/Cas9 lentivirus was packaged in 293T cells and used to infect bladder cells. The infected cells were selected by puromycin, and knockout efficiency was examined by western blot.

Sphere Formation
Bladder CSC self-renewal was detected by sphere-formation assay. The detected cells were incubated into DMEM/F12 sphere-formation medium, supplemented with 20 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL epidermal growth factor (EGF), N2, B27, and cultured in low-attached plate for 2 weeks' sphere formation. Spheres (diameter >500 mm) were counted, and sphere-formation ratios were calculated.

Transwell Invasion
Martigel-coated transwells were used for invasion assay. Bladder CSCs were seeded into the wells and cultured in FBS-free medium inside of the wells. FBS-supplemented medium was used outside of the well. 36 hr later, invasive cells that passed Martigel-coated membranes were stained with crystal violet.

Immunohistochemistry
5-µm bladder cancer sections were used for IHC. Samples were treated with xylene, gradient alcohol, and then treated with 3% H_2O_2 for 30 min. Antigen retrieval was performed using Tris-EDTA buffer for 15 min boiling. The samples were then incubated in Gprc5a primary antibody for 2 hr. After washing with PBS, horseradish peroxidase (HRP)-conjugated secondary antibody was added, and finally, DAB (diaminobenzidine) was used for DAB staining.

Real-Time PCR
RNA was extracted from bladder primary cells, CSCs, and spheres and then reverse-transcribed into cDNA. Gprc5a and circGprc5a primers were designed and obtained from the Sangon Company. For real-time PCR, bladder tumor cDNA was used as template, and the PCR reaction was performed using ABI7300.

Statistical Methods
GraphPad Prism 6, Adobe Photoshop CS6, and ImageJ were used for figure presentation. Excel 2010 was used for data analyses, and data are shown as mean ± SD. Two-tailed t test was used for statistical analysis: $*p < 0.05$, $**p < 0.01$, $***p < 0.01$, by unpaired one-tailed Student's t test; ns, not significant. For all panels in this work, at least three independent experiments were performed with similar results, and representative experiments are shown.

AUTHOR CONTRIBUTIONS
C.G. and N.Z. designed and performed experiments, analyzed data, and wrote the paper; Z.W. and G.L. performed experiments and analyzed data; Y.K., S.Y., Y.F., and L.C. performed some experiments; J.Y. initiated and analyzed data; F.T. initiated the study and organized, designed, and wrote the paper.

CONFLICTS OF INTEREST
The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

REFERENCES
1. Antoni, S., Ferlay, J., Soerjomataram, I., Znaor, A., Jemal, A., and Bray, F. (2017). Bladder Cancer Incidence and Mortality: A Global Overview and Recent Trends. Eur. Urol. *71*, 96–108.

2. Batlle, E., and Clevers, H. (2017). Cancer stem cells revisited. Nat. Med. *23*, 1124–1134.

3. Yang, Y.M., and Chang, J.W. (2008). Bladder cancer initiating cells (BCICs) are among EMA-CD44v6+ subset: novel methods for isolating undetermined cancer stem (initiating) cells. Cancer Invest. *26*, 725–733.

4. Zhu, D., Wan, X., Huang, H., Chen, X., Liang, W., Zhao, F., Lin, T., Han, J., and Xie, W. (2014). Knockdown of Bmi1 inhibits the stemness properties and tumorigenicity of human bladder cancer stem cell-like side population cells. Oncol. Rep. *31*, 727–736.

5. Jinesh, G.G., Choi, W., Shah, J.B., Lee, E.K., Willis, D.L., and Kamat, A.M. (2013). Blebbishields, the emergency program for cancer stem cells: sphere formation and tumorigenesis after apoptosis. Cell Death Differ. 20, 382–395.

6. Chen, Z., Zhu, P., Zhang, Y., Liu, Y., He, Y., Zhang, L., and Gao, Y. (2016). Enhanced Sensitivity of Cancer Stem Cells to Chemotherapy Using Functionalized Mesoporous Silica Nanoparticles. Mol. Pharm. 13, 2749–2759.

7. Takebe, N., Miele, L., Harris, P.J., Jeong, W., Bando, H., Kahn, M., Yang, S.X., and Ivy, S.P. (2015). Targeting Notch, Hedgehog, and Wnt pathways in cancer stem cells: clinical update. Nat. Rev. Clin. Oncol. 12, 445–464.

8. Chen, Z., Gao, Y., Yao, L., Liu, Y., Huang, L., Yan, Z., Zhao, W., Zhu, P., and Weng, H. (2018). LncFZD6 initiates Wnt/β-catenin and liver TIC self-renewal through BRG1-mediated FZD6 transcriptional activation. Oncogene 37, 3098–3112.

9. Chen, Z., Liu, Y., Yao, L., Guo, S., Gao, Y., and Zhu, P. (2018). The long noncoding RNA lncZic2 drives the self-renewal of liver tumor-initiating cells via the protein kinase C substrates MARCKS and MARCKSL1. J. Biol. Chem. 293, 7982–7992.

10. Zhu, P., Wang, Y., Wu, J., Huang, G., Liu, B., Ye, B., Du, Y., Gao, G., Tian, Y., He, L., and Fan, Z. (2016). LncBRM initiates YAP1 signalling activation to drive self-renewal of liver cancer stem cells. Nat. Commun. 7, 13608.

11. Zhu, P., Wang, Y., He, L., Huang, G., Du, Y., Zhang, G., Yan, X., Xia, P., Ye, B., Wang, S., et al. (2015). ZIC2-dependent OCT4 activation drives self-renewal of human liver cancer stem cells. J. Clin. Invest. 125, 3795–3808.

12. Chen, Z.Z., Huang, L., Wu, Y.H., Zhai, W.J., Zhu, P.P., and Gao, Y.F. (2016). LncSox4 promotes the self-renewal of liver tumour-initiating cells through Stat3-mediated Sox4 expression. Nat. Commun. 7, 12598.

13. Chen, Z., Yao, L., Liu, Y., and Zhu, P. (2018). LncTIC1 interacts with β-catenin to drive liver TIC self-renewal and liver tumorigenesis. Cancer Lett. 430, 88–96.

14. Zhu, P., Wang, Y., Huang, G., Ye, B., Liu, B., Wu, J., Du, Y., He, L., and Fan, Z. (2016). lnc-β-Catm elicits EZH2-dependent β-catenin stabilization and sustains liver CSC self-renewal. Nat. Struct. Mol. Biol. 23, 631–639.

15. Zhu, P.P., and Fan, Z.S. (2017). Cancer Stem Cell Niches and Targeted Interventions. Progress in Biochemistry and Biophysics 44, 697–708.

16. Memczak, S., Jens, M., Elefsinioti, A., Torti, F., Krueger, J., Rybak, A., Maier, L., Mackowiak, S.D., Gregersen, L.H., Munschauer, M., et al. (2013). Circular RNAs are a large class of animal RNAs with regulatory potency. Nature 495, 333–338.

17. Salzman, J. (2016). Circular RNA Expression: Its Potential Regulation and Function. Trends Genet. 32, 309–316.

18. Tian, M., Chen, R., Li, T., and Xiao, B. (2018). Reduced expression of circRNA hsa_circ_0003159 in gastric cancer and its clinical significance. J. Clin. Lab. Anal. 32, e22281.

19. Xu, L., Zhang, M., Zheng, X., Yi, P., Lan, C., and Xu, M. (2017). The circular RNA ciRS-7 (Cdr1as) acts as a risk factor of hepatic microvascular invasion in hepatocellular carcinoma. J. Cancer Res. Clin. Oncol. 143, 17–27.

20. Su, H., Lin, F., Deng, X., Shen, L., Fang, Y., Fei, Z., Zhao, L., Zhang, X., Pan, H., Xie, D., et al. (2016). Profiling and bioinformatics analyses reveal differential circular RNA expression in radioresistant esophageal cancer cells. J. Transl. Med. 14, 225.

21. Zheng, Q., Bao, C., Guo, W., Li, S., Chen, J., Chen, B., Luo, Y., Lyu, D., Li, Y., Shi, G., et al. (2016). Circular RNA profiling reveals an abundant circHIPK3 that regulates cell growth by sponging multiple miRNAs. Nat. Commun. 7, 11215.

22. Li, Z.Y., Huang, C., Bao, C., Chen, L., Lin, M., Wang, X.L., Zhong, G.L., Yu, B., Hu, W.C., Dai, L.M., et al. (2017). Corrigendum: exon-intron circular RNAs regulate transcription in the nucleus. Nat. Struct. Mol. Biol. 24, 194.

23. Legnini, I., Di Timoteo, G., Rossi, F., Morlando, M., Briganti, F., Sthandier, O., Fatica, A., Santini, T., Andronache, A., Wade, M., et al. (2017). Circ-ZNF609 Is a Circular RNA that Can Be Translated and Functions in Myogenesis. Mol. Cell 66, 22–37.e9.

24. Yang, Y., Fan, X., Mao, M., Song, X., Wu, P., Zhang, Y., Jin, Y., Yang, Y., Chen, L.L., Wang, Y., et al. (2017). Extensive translation of circular RNAs driven by N^6-methyladenosine. Cell Res. 27, 626–641.

25. Pamudurti, N.R., Bartok, O., Jens, M., Ashwal-Fluss, R., Stottmeister, C., Ruhe, L., Hanan, M., Wyler, E., Perez-Hernandez, D., Ramberger, E., et al. (2017). Translation of CircRNAs. Mol Cell 66, 9–21.e7.

26. Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A., and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. 8, 2281–2308.

27. Dorsam, R.T., and Gutkind, J.S. (2007). G-protein-coupled receptors and cancer. Nat. Rev. Cancer 7, 79–94.

28. Barker, N., van Es, J.H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P.J., and Clevers, H. (2007). Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449, 1003–1007.

29. Jaks, V., Barker, N., Kasper, M., van Es, J.H., Snippert, H.J., Clevers, H., and Toftgård, R. (2008). Lgr5 marks cycling, yet long-lived, hair follicle stem cells. Nat. Genet. 40, 1291–1299.

30. Ng, A., Tan, S., Singh, G., Rizk, P., Swathi, Y., Tan, T.Z., Huang, R.Y., Leushacke, M., and Barker, N. (2014). Lgr5 marks stem/progenitor cells in ovary and tubal epithelia. Nat. Cell Biol. 16, 745–757.

31. Huch, M., Dorrell, C., Boj, S.F., van Es, J.H., Li, V.S., van de Wetering, M., Sato, T., Hamer, K., Sasaki, N., Finegold, M.J., et al. (2013). In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. Nature 494, 247–250.

32. Zhu, P., Wang, Y., Du, Y., He, L., Huang, G., Zhang, G., Yan, X., and Fan, Z. (2015). C8orf4 negatively regulates self-renewal of liver cancer stem cells via suppression of NOTCH2 signalling. Nat. Commun. 6, 7122.

33. Meng, S., Zhou, H., Feng, Z., Xu, Z., Tang, Y., Li, P., and Wu, M. (2017). CircRNA: functions and properties of a novel potential biomarker for cancer. Mol. Cancer 16, 94.

34. Hansen, T.B., Jensen, T.I., Clausen, B.H., Bramsen, J.B., Finsen, B., Damgaard, C.K., and Kjems, J. (2013). Natural RNA circles function as efficient microRNA sponges. Nature 495, 384–388.

35. Gao, J., Xu, W., Wang, J., Wang, K., and Li, P. (2017). The Role and Molecular Mechanism of Non-Coding RNAs in Pathological Cardiac Remodeling. Int. J. Mol. Sci. 18, E608.

36. Morrison, C.D., Liu, P., Woloszynska-Read, A., Zhang, J., Luo, W., Qin, M., Bshara, W., Conroy, J.M., Sabatini, L., Vedell, P., et al. (2014). Whole-genome sequencing identifies genomic heterogeneity at a nucleotide and chromosomal level in bladder cancer. Proc. Natl. Acad. Sci. USA 111, E672–E681.