



Original Research Article

Circular RNA LMBR1 inhibits bladder cancer progression by enhancing expression of the protein ALDH1A3

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ARTICLE INFO

Keywords:

Bladder cancer
Chemical carcinogenesis
High-throughput sequencing
CircLMBR1
ALDH1A3

ABSTRACT

Background: Circular RNAs (circRNAs) have been identified as playing an integral role in the development of bladder cancer (BC). However, the mechanism by which circRNAs operate in the chemical carcinogenesis of BC remains unclear.

Methods: To explore this mechanism, we used RNA high-throughput sequencing to identify differentially expressed circRNA in bladder epithelial cells and chemically induced malignant transformed BC cells. Subsequently, *in vitro* experiments were conducted to investigate the biological function and molecular mechanism of circLMBR1 in BC. Finally, animal experiments were conducted to examine the clinical relevance of circLMBR1 *in vivo*.

Results: Our profiling of circular RNA expression during cellular malignant transformation induced by chemical carcinogens identified a subset of circRNAs associated with cell transformation. We verified that the expression of circLMBR1 in bladder epithelial malignant transformed cells was decreased compared with control cells, as well as in BC tissues and bladder cell lines. Furthermore, circLMBR1 was seen to inhibit the proliferation, invasion, and migration of BC cells both *in vitro* and *in vivo*. Mechanistically, circLMBR1 was found to exert its antitumor effect by binding to the protein ALDH1A3.

Conclusions: Our findings have revealed that circLMBR1 inhibits the progression of BC cells by binding to ALDH1A3 and upregulating its expression. As such, circLMBR1 serves as a promising predictor of BC and may provide a novel therapeutic target for the treatment of BC.

1. Introduction

Bladder cancer (BC) is a devastating condition and one of the most commonly occurring malignant tumors in the genitourinary system. According to medical literature, cancer is the leading cause of death in China in 2020, with bladder cancer ranking 10th in the world [1]. Its incidence is believed to be the result of a combination of genetic and external factors. Internally, genetic factors such as point mutations,

rearrangements, deletions, and chromosome abnormalities can activate proto-oncogenes and deactivate suppressor genes [2]. Externally, smoking and exposure to chemical carcinogens such as aniline, diamminobiphenyl, and 1-naphthylamine can contribute to carcinogenesis [3]. Unfortunately, the exact molecular mechanism of bladder cancer caused by chemical exposure remains unclear. Due to its high mortality rate, high recurrence rate, and expensive treatment cost, there is an urgent need to explore bladder cancer biomarkers in order to advance

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research and prolong patient survival [4,5].

Circular RNA (circRNA) is a newly identified endogenous non-coding RNA (ncRNA) that was originally thought to be an incidental by-product [6]. Different from its traditional linear RNA counterpart, circRNA has a closed loop structure without a 5' cap or 3' poly A tail [7,8]. Studies have revealed that circRNA has a wealth of characteristics, such as stable structure, conserved sequence, and cell or tissue-specific expression [9–11]. These findings suggest that circRNA formation is a regulatory process, not simply an outcome of splicing errors, and may play a role in regulating gene expression. It has been reported that there is a close association between circRNA and various types of tumors [12]. Some circRNAs contain miRNA response elements (MRE), indicating that they can act as endogenous RNAs (ceRNAs) to compete with miRNA binding sites, thereby affecting miRNA activity [13,14]. Additionally, circRNA has been linked to protein, such as sponging or acting as a bait or scaffold for biological regulation in certain tumors [15–17]. For example, circ_0000658 down-regulates miR-498 by up-regulating HMGA_2 to increase the virulence of bladder cancer cells [18]. CircSEMA4B encodes a novel protein SEMA4B-211aa and sponges miR-330-3p to suppress the progression of breast cancer [19]. CircRNA is also strongly correlated to the progression of bladder cancer [20–22]. However, the extent to which circRNA can modulate the regulation of RNA-binding proteins in bladder cancer, and its regulatory mechanism, remain to be elucidated.

In this study, we identified a circRNA derived from the LMBR1 gene, named circLMBR1, through high-throughput sequencing analysis of two groups of chemically-induced malignant transformed bladder epithelial cells and one group of normal bladder epithelial cells. CircLMBR1 expression was found to be decreased in bladder cancer cells. Functional studies demonstrated that circLMBR1 can inhibit bladder cancer cell progression both *in vitro* and *in vivo*. Subsequent studies revealed that circLMBR1 suppresses the proliferation, cloning, invasion, and migration of bladder cancer cells by binding to the protein ALDH1A3 and up-regulating its expression. Our results demonstrate that circLMBR1 is a promising diagnostic tool and therapeutic target for bladder cancer.

2. Materials and methods

2.1. Cell culture and treatment

Human bladder epithelial immortalized cells (SV-HUC-1), 293T cells and trimethylcholine-induced human bladder epithelial immortalized cells (MCA-SV-HUC-1) were procured from American Type Culture Collection (ATCC, USA). The human BC cell lines, for example, T24, 5637, UM-UC-3 and J82 were retrieved from Shanghai Type Collection of Chinese Academy of Sciences (Shanghai, China). Cadmium chloride-induced human bladder epithelial immortalized cells (CdCl₂-SV-HUC-1) were previously established in our laboratory. CdCl₂-SV-HUC-1 and MCA-SV-HUC-1 were cultured with F-12K medium (Gibco, USA), J82 and UM-UC-3 were cultured with MEM medium (Gibco, USA), T24 and 5637 were cultured with RPMI 1640 medium (Gibco, USA), whereas 293T was cultured with DMEM medium (Gibco, USA). All the aforementioned media were supplemented with 10 % fetal bovine serum (Gibco, USA) and 1 % penicillin/streptomycin (Gibco, USA). All the cells mentioned above were cultured in incubators containing 5 % CO₂ and a set temperature of 37 °C.

2.2. RNA extraction and qRT-PCR

Total RNA of cultured cells was extracted using Trizol reagent (Invitrogen, USA) in accordance with the manufacturer's instructions. Subsequently, quality control and concentration of the sample were assessed using Nanodrop2000 spectrophotometer. cDNA was synthesized by using Primescript™ RT reagent Kit with gDNA Eraser (Takara, Japan) with 1 µg of RNA. qRT-PCR was performed using SYBR® Premix Ex Taq™ Kit (Takara, Japan). Each sample was analyzed in triplicate,

and GAPDH was used as an internal control. The relative expression of different genes was determined by calculating the $2^{-\Delta\Delta C_t}$ value. The primers used are listed in Table S1.

2.3. CircRNA sequencing and bioinformatic analysis

In order to enrich circRNA, DNase I was employed for the removal of DNA, and RNase R was used to eliminate linear RNA. A specific cDNA library was constructed and purified by magnetic beads. The sequencing was conducted using the Illumina HiSeq™2000 platform, and the raw data was filtered by FastQC software to obtain clean data for further analysis. BWA software was utilized to compare the sequence with the reference genome, and CIRI software was used to identify the circRNA of sequencing samples. Subsequently, the mutually down-regulated and up-regulated circRNA in malignant transformed cells were filtered.

2.4. Protein preparation and western blot

Cellular proteins were isolated using RIPA Buffer (Sigma-Aldrich, USA) containing loading buffer, DL-Dithiothreitol, Phenylmethanesulfonyl fluoride, and Phosphate Buffered Saline, and lysed on ice for 10 min. The protein extracts were then heated in a metal bath at 100 °C for 5 min before being separated by SDS-PAGE and transferred onto PVDF membranes (Sigma-Aldrich, USA). After blocking for 1 h with 5 % BSA (Sigma-Aldrich, USA) in Tris-buffered saline containing 0.1 % Tween 20 (TBST), the membranes were incubated with each primary specific antibodies [GAPDH (Cell Signaling Technology, # 3907), ACTA2 (Cell Signaling Technology, #19245), ALDH1A3 (Abcam, ab308526), AKR1B1 (Proteintech, Cat No.15439-1-ap), LAMB3 (Proteintech, Cat No.26795-1-AP)] at 4 °C overnight. Subsequently, they were reacted with corresponding secondary antibodies for 2 h and developed with chemiluminescent substrates.

2.5. Transfections and plasmid constructions

To investigate the role of circLMBR1 *in vitro*, a 578 bp cDNA fragment containing the circLMBR1 gene was cloned into a Dendritic poly-L-lysine (PLL) vector (Thermo Scientific, USA) between Not I and Xho I restriction sites. To evaluate the effects of circLMBR1 knockdown, cells were transfected with siRNAs (IGE Biotechnology Co., Ltd., Guangzhou, China) targeting circLMBR1. siRNA sequences are listed in Table S2. Plasmids or siRNAs were transfected into cells using Lipofectamine 3000 Reagent (Invitrogen, USA) or Lipofectamine RNAiMAX Reagent (Invitrogen, USA) respectively, according to the manufacturer's protocols. ALDH1A3 knockout was performed as previously described [23].

2.6. RNA pulldown and mass spectrometry

To elucidate the molecular partners of circLMBR1, the Pierce™ Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher, USA) was employed for a circRNA pulldown assay. The oligonucleotide probe modified by biotin was designed and synthesized based on the junction region of circLMBR1 (IGE Biotechnology Co., Ltd., Guangzhou, China): 5'-GTGAACGTGCTCAAAAACAACCATTAGTCGTCTCTGGAGTG-3'. At least 10⁷ cells were lysed in each group, and then 50 µl of streptavidin magnetic beads washed with 20mMTris was incubated with the cell lysate and 100 nmol probe at room temperature for 30 min. Subsequently, the unbound RNA was washed away using 20 mM Tris, and 100 µl 1 × RNA-protein binding buffer with 100 µg total protein was added to the Streptavidin Magnetic Beads. After rotating and incubating at 4 °C for 60 min, Streptavidin Magnetic Beads were washed with washing buffer for 3 times. The supernatant was collected after incubating the beads with 50 µl elution buffer at 37 °C for 30 min, and then analyzed by Coomassie brilliant blue staining and mass spectrometry.

2.7. RNA-FISH

To further verify the subcellular localization of circLMBR1, a Cy3-labeled specific probe for circLMBR1 was synthesized by Guangzhou Ruibo Biotechnology Co., Ltd. (sequence: 5'-cy3-GTGAACGTGCTCAAAAACAACCATTTAGTCGTCCTCTGGAGTG-3'). The signal was detected using the FISH kit (Ruibo). Cells were fixed with 4 % paraformaldehyde (PFA) for 20 min at 25 °C when the cell fusion reached 40–50 %. After osmosis (1 × PBS/0.2% Triton X-100), the specific probe of circLMBR1 was hybridized with ACTA2 antibody (Cell Signaling Technology Cat# 19245, RRID: [AB_2734735](#)) and ALDH1A3 antibody (Abcam Cat# ab80176, RRID: [AB_1603395](#)) overnight in hybridization buffer at 37 °C. Subsequently, the hybridization buffer was gradually eluted with 2 × SSC (containing 0.1 % Tween-20), 1 × SSC and 0.5 × SSC at 42 °C, and 6-diamino-2-phenylindole (DAPI) (RiboBio) was used to counterstain the nucleus. Images were captured using the LAS X software and Leica TCS SP8 STED confocal microscope system (Leica, Germany).

2.8. Cell proliferation assay (MTS) and colony formation assay

To assess the influence of overexpressed circLMBR1 on MCA-SV-HUC-1 and CdCl₂-SV-HUC-1 and the influence of low-expressed circLMBR1 on SV-HUC-1, cell proliferation and colony formation assays were employed. For the MTS assay, MCA-SV-HUC-1 and CdCl₂-SV-HUC-1 (overexpressed circLMBR1) and SV-HUC-1 (low-expressed circLMBR1) were seeded in 96-wells plates at a density of 1000–5000 cells per well, and the OD values at 490 nm were then recorded using an Automatic enzyme labeling instrument (Bio Tek, USA). For the colony formation assay, MCA-SV-HUC-1 and CdCl₂-SV-HUC-1 (overexpressed circLMBR1) and SV-HUC-1 (low-expressed circLMBR1) were cultured in 6-wells plates at the same density as the MTS assay, and incubated at 37 °C with 5 % CO₂ for 2–3 weeks. After the visible formation of colonies, the cells were immobilized with PFA and stained with 1 % crystal violet. The number of colonies was then photographed for counting.

2.9. Wound healing assay

To evaluate the effect of circLMBR1 on cell migration, a wound healing assay was employed using MCA-SV-HUC-1, CdCl₂-SV-HUC-1 and SV-HUC-1 with plasmids. The cells were seeded into 96-wells plates at a density of 1 × 10⁵ cells per well, and wounds were then made by a Cell player 96-wells woundmaker (Essen BioScience, USA). After removing any residual cells and medium with PBS, 1 % serum was added. The migration ability of the cells was then observed using an IncuCyte Zoom (Essen BioScience, USA). Images were taken once an hour for a total of 24 or 36 h, and Relative Wound Density (Percent) was calculated for later analysis.

2.10. Matrigel invasion assay

The matrigel invasion assay was conducted to determine the effect of circLMBR1 on cell invasion. 10 mg/mL Matrigel matrix (Corning Cat. No.354234) was mixed with medium at a ratio of 1:10, and 50 µL of the diluted matrix was added to each chamber and cultured at 37 °C for 2 h. After 2 h, 1 × 10⁵ cells were inoculated into the upper chamber and 200 µL of serum-free medium was added. 500 µL of 100 % serum was then added to the bottom chamber. After 14–16 h, the invaded cells were washed with PBS, fixed with PFA for 10 min, and then stained with 0.1 % crystal violet at room temperature for 30 min. The number of invaded cells was counted under a 200x high power lens (ZEISS Axio Imager.Z2 microscope, Germany). Five visual fields were randomly selected, and the average value was then taken.

2.11. RNA binding protein immunoprecipitation (RIP) assay

To conduct the RIP assay, we followed the established protocol [24]. As a negative control, normal rabbit IgG was used, while anti-ACTA2 and anti-ALDH1A3 were utilized as positive controls. Subsequently, qRT-PCR was employed to analyze the isolated RNA obtained from the RIP assay.

2.12. Xenograft tumor models

The animal assays were performed in accordance with the protocol approved by the Ethics Committee of Guangzhou Medical University (2018-192). We procured the 5-week-old BALB/c mice from Guangdong Medical Experimental Animal Center, and the transgenic mutant zebra fish fertilized eggs were sourced from the zebra fish experimental platform of the Medical College of Sun Yat-sen University. Zebrafish were raised at Sun Yat-sen University. Tumor cells were stained with Cell-Tracker™ CM-DiI dye (Invitrogen, Cat.#: C7000). The cells were injected into the yolk sac or blood vessels of 2-day-old zebrafish embryos using stereoscopic microinjection technology. The zebrafish embryos were continuously observed for 7 days and the number of cells transferred to the tail perforating blood vessels of zebrafish embryos was recorded. Images of the zebrafish were taken using a Zeiss Lumar 12 stereoscopic microscope and light sheet microscope (Carl Zeiss, Germany). Subcutaneous implantation: 8 × 10⁶ cells were injected subcutaneously into nude mice, and the formation and growth of tumors were measured weekly. After 4 weeks, the mice were euthanized, and the tumors were weighed. Intraperitoneal injection of 3 % pentobarbital sodium is administered to the animal, and cervical dislocation is performed after deep coma. Pentobarbital sodium is dissolved in physiological saline, and the injection volume is 0.1 mL per 100g. The tumor volume = length x width x 0.52.

2.13. Statistical analysis

We employed SPSS 22.0 and GraphPad Prism 8.0 software for statistical analysis. All data were repeated at least three times and expressed as mean ± standard error. To compare two independent samples obeying the positive distribution, we used Student's *t*-test. For two independent samples disobeying the positive distribution, we conducted Wilcoxon Rank sum test of non-parametric test. All tests were done at an inspection level of $\alpha = 0.05$, and $P < 0.05$ was considered to be statistically significant, denoted with an asterisk (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

3. Results

3.1. Changes in CircRNA expression profile during malignant transformation of bladder epithelium

The high throughput sequencing of SV-HUC-1, MCA-SV-HUC-1 and CdCl₂-SV-HUC-1 revealed a total of 8929 circRNAs, mainly from exons, which were distributed across all chromosomes, with the highest number of circRNAs in chromosome 1 (Fig. 1A and B). In SV-HUC-1, 3885 circRNAs were identified, of which 2334 were found in circBase and the remaining 1551 were newly discovered. Similarly, 2759 and 4970 circRNAs were identified in MCA-SV-HUC-1 and CdCl₂-SV-HUC-1 respectively, with 1554 and 3436 circRNAs being compared in circBase (Table S3).

To investigate the changes in circRNA expression during malignant transformation of bladder epithelium, we identified the significantly differentially expressed circRNAs (FDR ≤ 0.001 and |log₂Ratio| ≥ 1) between the two groups before and after malignant transformation. We observed 2058 circRNAs to be up-regulated and 2685 to be significantly down-regulated in trimethylcholanthracene-induced human bladder epithelial cells compared to normal human bladder epithelial cells.

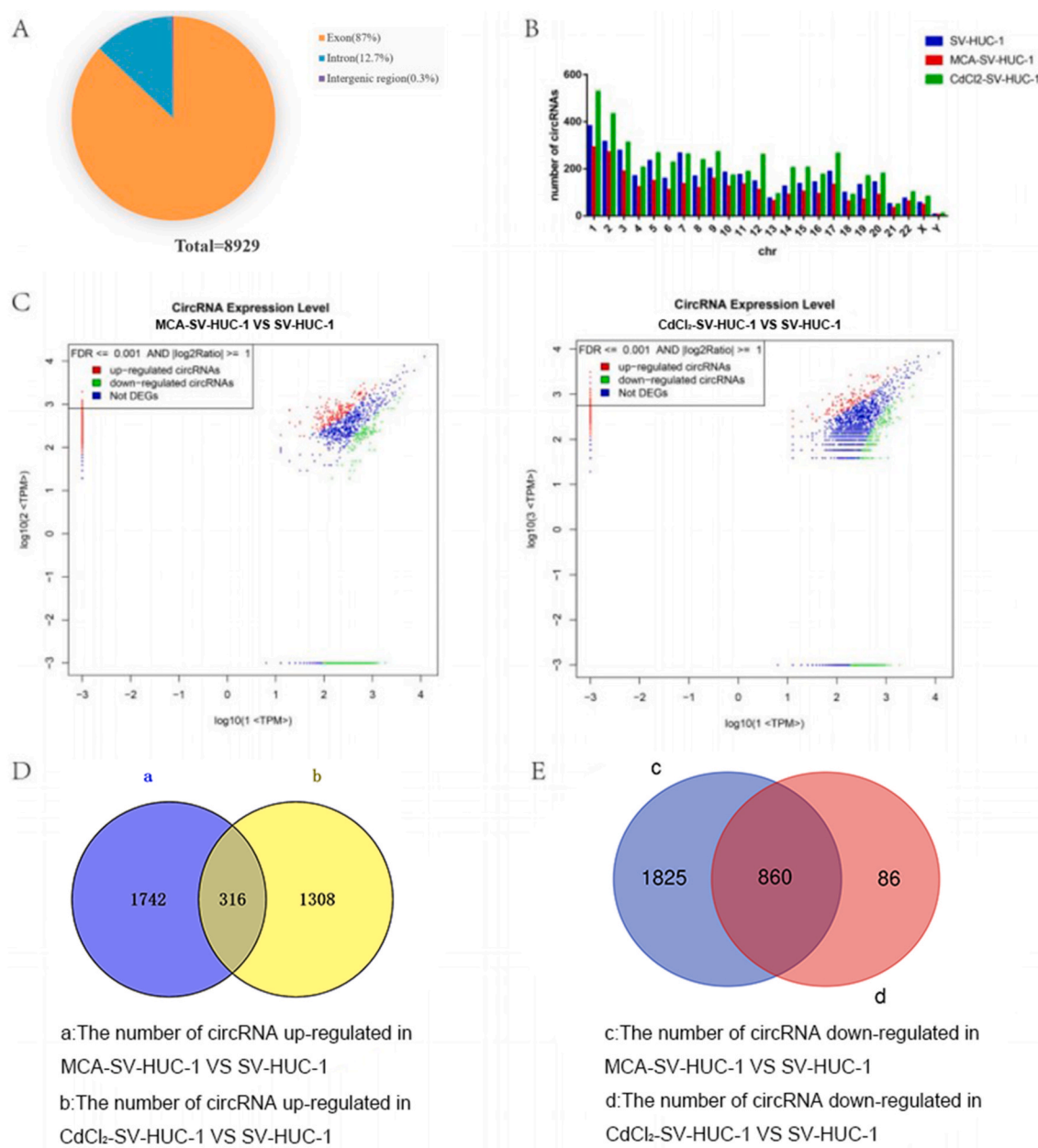


Fig. 1. Changes of circRNA expression profile in malignant transformation of bladder epithelial cells. **A.** Source of circRNA. **B.** Distribution of circRNA in chromosomes. **C.** Expression of circRNA in normal and malignant bladder epithelial cells. **D.** Commonly up-regulated circRNAs in two malignant transformed cells. **E.** Commonly down-regulated circRNAs in two malignant transformed cells.

Similarly, 1624 circRNAs were observed to be up-regulated and 946 significantly down-regulated in cadmium chloride-induced human bladder epithelial cells relative to normal human bladder epithelial cells (Fig. 1C). By comparing the two groups, 316 were up-regulated and 860 were down-regulated (Fig. 1D and E). These data suggest that the circRNA expression profile of chemically induced malignant transformed cells is distinct from that of immortalized human bladder epithelial cells, thus providing key insights into the malignant transformation of bladder epithelium.

3.2. Verification of circRNA and expression and localization of circLMBR1

We selected five common differential circRNAs (Table S4) to verify the reliability of our sequencing results. To do this, we designed specific

outward primers for the junction site, followed by PCR amplification and agarose gel electrophoresis. We found that circGFM2(hsa_circ_0129604), circLMBR1(hsa_circ_0003511) and circORC4(hsa_circ_0001047) could run out of target bands of the corresponding size (Fig. 2A). To confirm our results, we recovered the target bands of circGFM2, circORC4 and circLMBR1 and sent them to Guangzhou IGE Biotechnology Co., Ltd. for Sanger sequencing. This revealed that the target bands were indeed PCR products containing the target circRNA junction sequence (Fig. 2B). This evidence provided a foundation for further assays and demonstrated the reliability of our circRNA sequencing results.

In the subsequent experimental verification, only circLMBR1 was found to be consistent with the sequencing results in the previous section. Through searching existing databases, it was found that only circLMBR1 may have biological functions(<https://circinteractome.irp>.

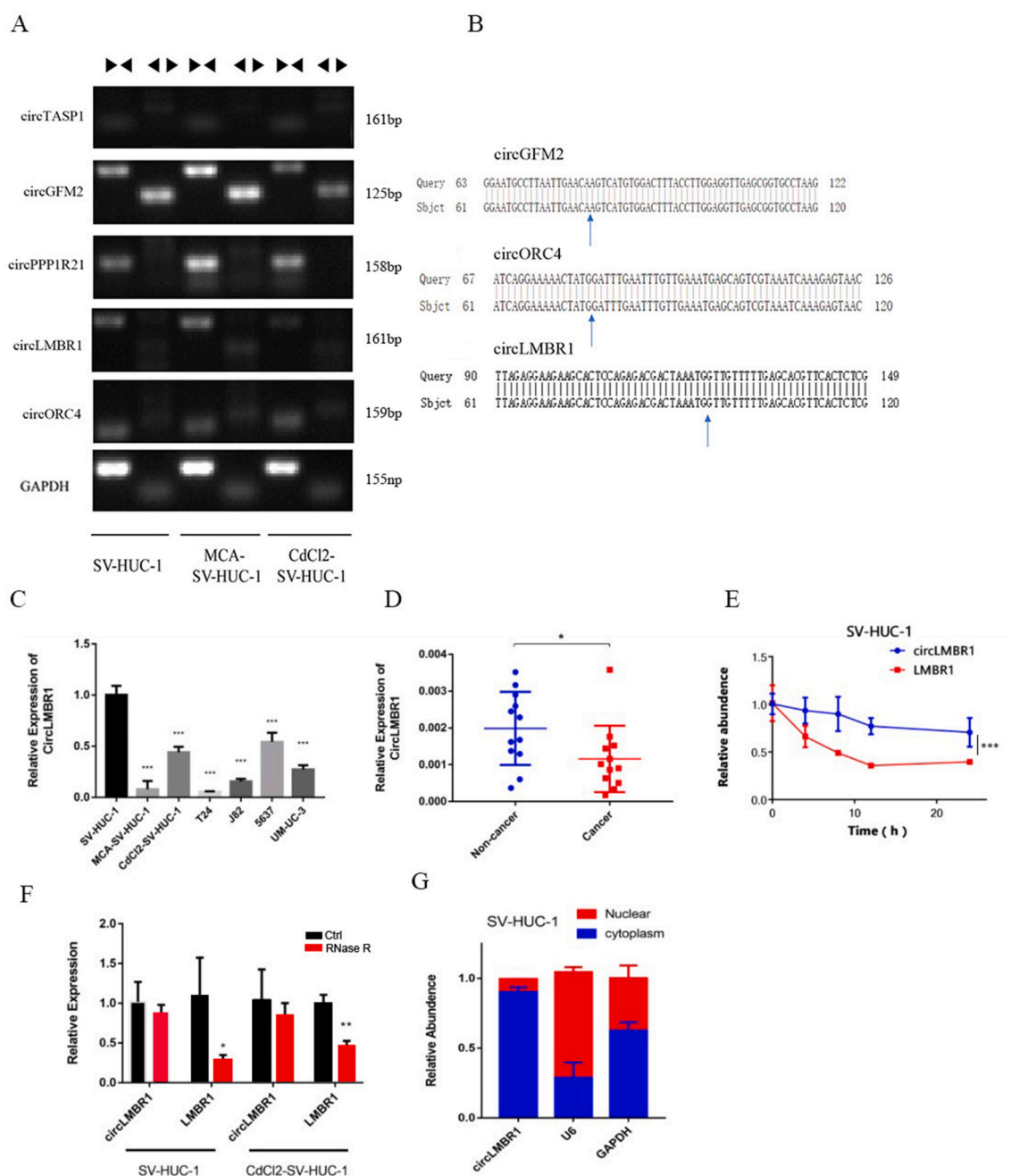


Fig. 2. Validation of circRNA and expression of circLMBR1 in normal and bladder cancer cells and tissues. **A.** Verification results of circRNAs agarose gel electrophoresis. ►◄ indicates convergent primers and ◄► indicates divergent primers. **B.** Sanger Sequencing result of PCR products. Blue arrows indicate the back-splicing site, which is a characteristic of circular RNAs. **C.** Expression of circLMBR1 in normal human uroepithelial SV-HUC-1 cells, bladder epithelial malignant transformed cell lines and BC cell lines, measured by qRT-PCR. **D.** Expression of circLMBR1 in bladder tumors was lower than that in normal tissues, as determined by qRT-PCR ($n = 12$, $P < 0.05$). **E.** Cells treated with actinomycin D were collected at different time points (0,4,8,12 and 24 h), and the expression of circLMBR1 and LMBR1 was detected by qRT-PCR. **F.** After RNase R treatment, the expression of circLMBR1 and LMBR1 mRNA was detected by qRT-PCR. **G.** qRT-PCR analysis of circLMBR1 in nuclear and cytoplasmic fractions of SV-HUC-1 cells, showing that circLMBR1 is mainly located in the cytoplasm. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

nia.nih.gov/). So we chose circLMBR1 for subsequent experiments. We previously designed specific primers for circLMBR1 according to its junction site, and verified its existence by PCR, agarose gel electrophoresis and Sanger sequencing. We then detected the expression of circLMBR1 in SV-HUC-1, malignant cell lines and BC cell lines by qRT-PCR. Compared to SV-HUC-1, the expression of circLMBR1 was significantly lower in malignant cell lines and BC cell lines ($P < 0.001$), especially in T24 and MCA-SV-HUC-1 (Fig. 2C).

We further extracted RNA from 12 pairs of clinical cancer and paracancerous tissues by Trizol and detected the expression of circLMBR1

by qRT-PCR. Our results showed that the expression of circLMBR1 in BC was lower than that in paracancerous tissues ($P < 0.05$) (Fig. 2D). This stability of circLMBR1 is likely attributed to its lack of a 3'-terminal adenylate tail, which would otherwise render it vulnerable to degradation by RNA. To confirm this hypothesis, we treated SV-HUC-1 cells with actinomycin D to inhibit transcription and found that the half-life of circLMBR1 was longer than 24 h, while the expression of LMBR1 mRNA decreased to less than 50 % within 10 h (Fig. 2E). We then treated the RNA of SV-HUC-1 and Cdc12-HUC-1 with RNaseR and detected the expression of circLMBR1 and LMBR1 mRNA by qRT-PCR. Our results

showed that there was no significant change in the expression of circLMBR1 before and after RNaseR treatment, but the expression of LMBR1 mRNA decreased significantly after RNA enzyme treatment (Fig. 2F), further confirming the presence of circLMBR1 in the cell line. In addition, we assessed the expression of circLMBR1 in the cytoplasm and nucleus by qRT-PCR. We compared the expression of circLMBR1 to U6 and GAPDH and found that circLMBR1 was primarily localized in the cytoplasm of bladder epithelial cells (Fig. 2G).

3.3. Overexpression of CircLMBR1 inhibits malignant transformation

Our results demonstrated that circLMBR1 is significantly down-regulated in malignant transformed cells and tumor cells and tissues, indicating that it may serve as a tumor suppressor gene. To further investigate this hypothesis, we overexpressed circLMBR1 in MCA-SV-HUC-1 and CdCl₂-SV-HUC-1 cells, and the increased expression of circLMBR1 was detected by qRT-PCR (Fig. 3A). Transwell assay showed that overexpression of circLMBR1 significantly inhibited the invasive ability of MCA-SV-HUC-1 and CdCl₂-SV-HUC-1 cells compared with the control conditions (Fig. 3B). In addition, the proliferation and colony formation assay showed that after overexpressing circLMBR1, the proliferation and colony formation ability of MCA-SV-HUC-1 and CdCl₂-SV-HUC-1 cells were significantly reduced compared to the control group (Fig. 3C and D). Furthermore, the wound healing assay revealed that overexpression of circLMBR1 could significantly slow down the migration ability of cells ($P < 0.05$) (Fig. 3E).

3.4. Downregulation of CircLMBR1 promotes bladder epithelial cell proliferation and migration

To further explore the role of circLMBR1 in bladder epithelial cells, we designed siRNA according to the junction site of circLMBR1 and transfected it into SV-HUC-1 cell. The expression of circLMBR1 was verified by qRT-PCR, and no significant change in LMBR1 mRNA expression was observed (Fig. 4A). Subsequent experiments revealed that when si-circLMBR1 was transfected into SV-HUC-1 cells, their ability of cell proliferation was enhanced compared to the control group (Fig. 4B). Colony formation assay also showed that knocking down circLMBR1 could significantly increase the proliferation ability of SV-HUC-1 cells ($P < 0.01$) (Fig. 4C). Similarly, wound healing assay demonstrated that knocking down circLMBR1 could significantly enhance the migration ability of SV-HUC-1 cells ($P < 0.05$) (Fig. 4D).

3.5. Overexpression of CircLMBR1 inhibits subcutaneous growth and metastasis of BC cells

To further validate the role of circLMBR1 *in vivo*, we constructed subcutaneous tumor model in nude mice and a tail metastasis model in zebrafish. We constructed a T24 cell line stably overexpressing circLMBR1, and the increased expression of circLMBR1 was verified by qRT-PCR (Fig. 5A). Subsequently, T24 cells with overexpression of circLMBR1 and empty vector were injected into nude mice, respectively. The results showed that overexpression of circLMBR1 could significantly decrease the size and weight of subcutaneous tumors compared with the control group (Fig. 5B and C). Moreover, when CdCl₂-SV-HUC-1 cells with overexpression of circLMBR1 and empty vector were injected into the yolk sac of zebrafish by microinjection system, the results showed that overexpression of circLMBR1 could significantly reduce the number of cells transferred to the caudal vessels of zebrafish ($P < 0.01$) (Fig. 5D). Overall, the experimental results from *in vitro* and *in vivo* models suggest that circLMBR1 could inhibit the malignant transformation, proliferation and metastasis of bladder epithelial cells.

3.6. CircLMBR1 can bind to ACTA2 and ALDH1A3 proteins

We carried out RNA pulldown assay on MCA-SV-HUC-1 and CdCl₂-

SV-HUC-1 cells. After getting the eluent, we sent it to Hangzhou Jingjie Biotechnology Co., Ltd for mass spectrometry identification. The results showed that, compared to the control group, the number of proteins up-regulated and down-regulated in CdCl₂-SV-HUC-1 cells was 59 and 54 respectively, while in MCA-SV-HUC-1 cells, the number of proteins up-regulated and down-regulated in probe groups was 95 and 123 respectively (Fig. 6A). The four proteins identified as having the most binding amount in CdCl₂-SV-HUC-1 and MCA-SV-HUC-1 cells were ACTA2, AKR1B1, ALDH1A3 and LAMB3. To further verify the accuracy of the results, we repeated the RNA pulldown assay and Western blot assay with the eluent, and the results confirmed that ACTA2, ALDH1A3 and AKR1B1 were successfully pulled down by the probe (Fig. 6B). As an additional measure of reliability, we conducted RIP assays using antibodies against ACTA2 and ALDH1A3 to enrich the RNA bound to them. Then, qRT-PCR revealed that the expression level of circLMBR1 enriched with ACTA2 and ALDH1A3 proteins was significantly increased compared to the control group (Fig. 6C). To assess the co-localization of circLMBR1 and protein ACTA2 and ALDH1A3, we designed a circLMBR1 probe with fluorescence labeling and conducted a FISH assay, confirming the co-localization of circLMBR1 and protein ACTA2 and ALDH1A3 in the cytoplasm (Fig. 6D).

3.7. ALDH1A3 expression is regulated by CircLMBR1 and inhibits BC progression

To further investigate the effect of circLMBR1 expression on protein ACTA2 and ALDH1A3, we overexpressed and knocked down circLMBR1 in malignant transformed cells and conducted Western blot assays. The results showed that overexpression of circLMBR1 could up-regulate the expression of protein ALDH1A3 (Fig. 7A). This result suggests that circLMBR1 may inhibit the invasion, proliferation, cloning, and migration of malignant transformed cells by up-regulating the expression of protein ALDH1A3. To test this theory, we used CRISPR online design website (<http://chopchop.cbu.uib.no/>) to design three pairs of sgRNA for ALDH1A3, constructed a malignant transformed cell stable strain by lentivirus packaging, and verified its knockdown effect by Western blot assay (Fig. 7B). We then selected the third pair for further assays. Through transwell, proliferation, colony formation and wound healing assays, we found that in the malignant transformed cells overexpressing circLMBR1, the ability of invasion, proliferation, cloning and migration of malignant transformed cells was restored after knocking down ALDH1A3 (Fig. 7C–F). This indicates that knocking down ALDH1A3 can reverse the function of overexpressing circLMBR1 in inhibiting the invasion, proliferation, cloning and migration of malignant transformed cells.

3.8. Knockdown of ALDH1A3 reverses the inhibitory function of CircLMBR1 on subcutaneous growth

To further explore the function of circLMBR1 and protein ALDH1A3 *in vivo*, we constructed a subcutaneous tumor model in nude mice. We constructed two T24 cell lines, overexpressing circLMBR1 and knocking down ALDH1A3 in overexpressing circLMBR1. qRT-PCR and Western blot experiments verified the expression of circLMBR1 and ALDH1A3 (Fig. 8A and B). Two groups of T24 cells were subcutaneously injected into nude mice. Results showed that in T24 cells overexpressing circLMBR1 and knocking down ALDH1A3 could significantly increase the size and weight of subcutaneous tumors in nude mice ($P < 0.05$) (Fig. 8C and D). Overall, our findings suggest that circLMBR1 and protein ALDH1A3 play a critical role in the progression of BC, and knock-down of ALDH1A3 can reverse the inhibitory function of circLMBR1 on subcutaneous growth.

4. Discussion

The unique structure of circular RNA (circRNA) is formed by reverse

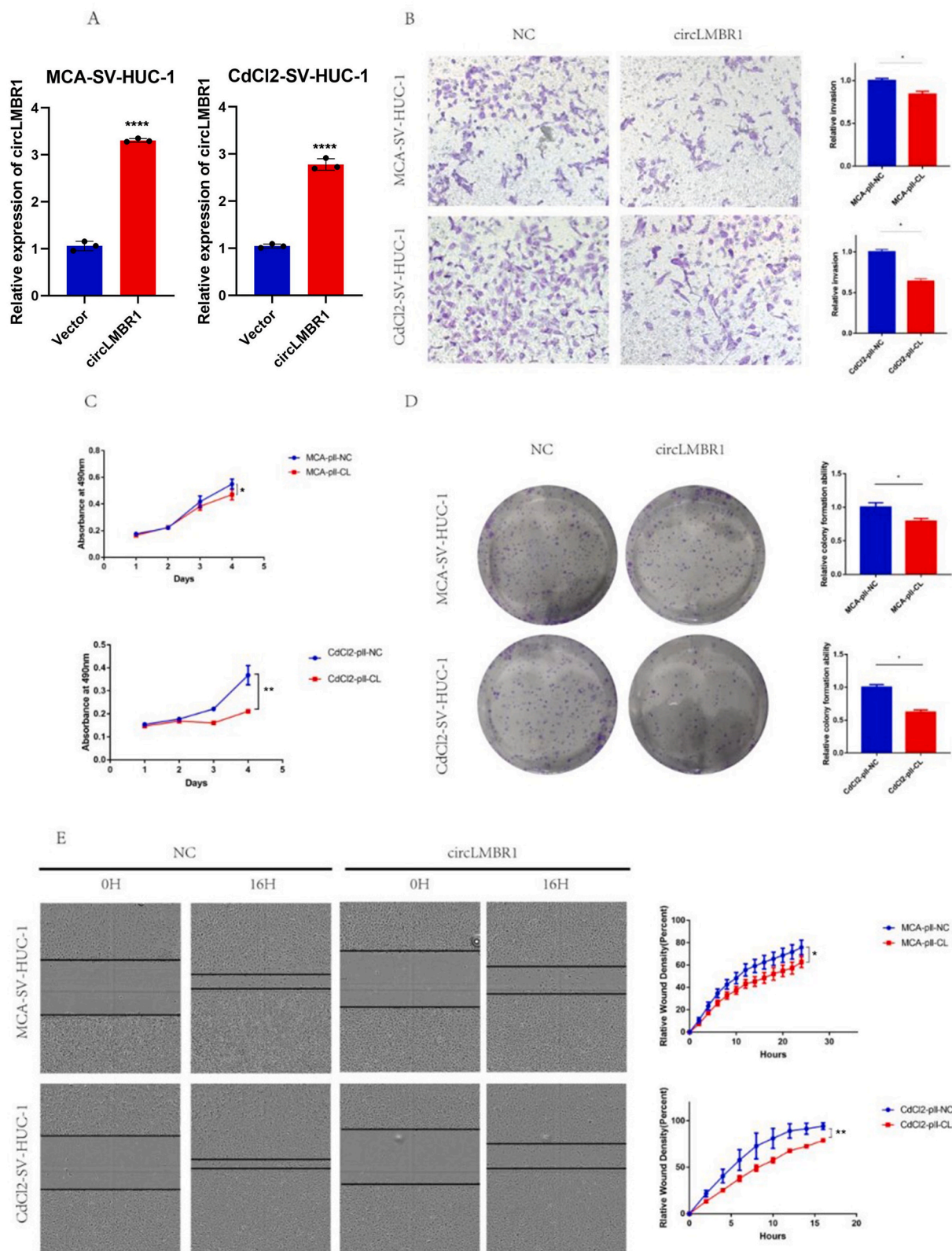


Fig. 3. Overexpression of circLMBR1 can inhibit the proliferation, invasion and migration of malignant transformed cells. **A.** After constructing stable cell line, the expression of circLMBR1 was detected by qRT-PCR. **B.** The effect of overexpression of circLMBR1 on cell invasion was analyzed by Transwell assay, 200x magnification. **C, D.** The effect of overexpression of circLMBR1 on cell proliferation was analyzed by MTS assay and colony formation assay. **E.** The effect of overexpression of circLMBR1 on cell migration was analyzed by wound healing assay, 4 × magnification. Statistical significance was assessed by Two-way ANOVA; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

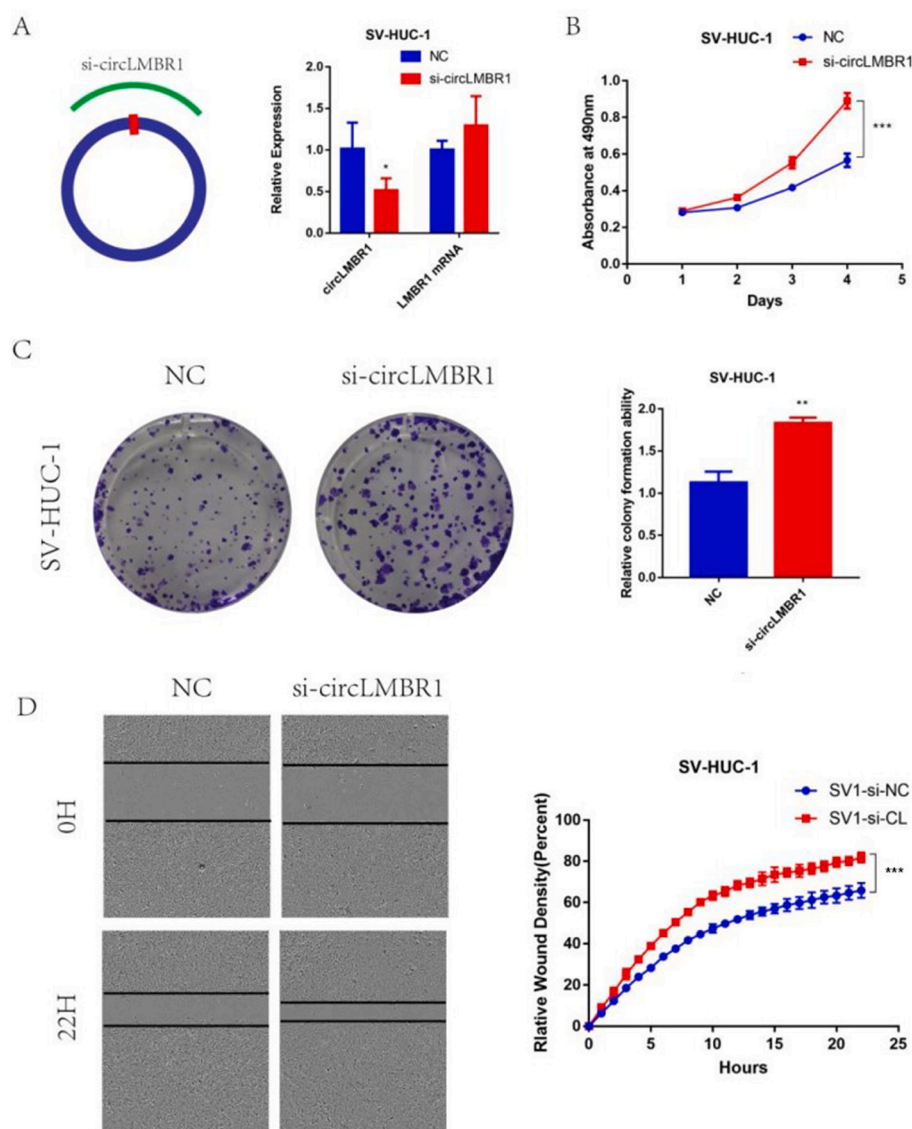


Fig. 4. Effects of knocking down circLMBR1 expression on bladder epithelial cell proliferation and migration. **A.** The design site of si-circLMBR1 and the expression of circLMBR1 were detected by qRT-PCR. **B.** The effect of knocking down circLMBR1 on cell proliferation was analyzed by MTS assay. **C.** The effect of knocking down circLMBR1 on cell proliferation was analyzed by colony formation assay. **D.** Analysis of the effect of tapping circLMBR1 on cell migration by wound healing assay, 4 × ; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

shear [25]. This structure has enabled it to attract immense attention in recent years due to its high expression, high stability, and high specificity. Studies have demonstrated the involvement of circRNAs in the onset and progression of numerous diseases, including atherosclerotic vascular disease, nervous system disorders, and endocrine diseases [26]. It is reported that circKIF4A-miR-637-STAT3 axis promotes brain metastasis of triple negative breast cancer [27]. In another study, CircUCP2 promoted tumor progression in non-small cell lung cancer through the miR-149/UCP2 pathway [28]. Similarly, in prostate cancer, downregulation of circROBO1 has been shown to significantly inhibit the proliferation of prostate cancer cells [29]. Furthermore, circRNAs have been found to be closely associated with the development of several types of tumors [30–33].

Carcinogenic chemicals are known to play a role in the onset and progression of tumors. However, comparatively few studies have been conducted to elucidate the relationship between chemical carcinogenesis and circRNA regulation. It has been reported that circRNA 100146 promotes cell proliferation and invasion in malignant transformed human bronchial epithelial cells induced by anti-benzopyrene-trans-7,8-

dihydrodiol-9-and 10-epoxide, while silencing circ0006916 promotes cell progression [34,35]. Similarly, it has been reported that circ100284 accelerates the cell cycle progression of human keratinocytes during malignant transformation with arsenite [36].

The functional role and molecular mechanism of circRNA in chemically induced BC remain largely unknown. We used two carcinogens, the chemical carcinogen methylcholanthrene and the metal carcinogen cadmium chloride, to perform malignant transformation on human bladder epithelial cells. Our high-throughput sequencing data revealed some new circRNA expression profiles in BC cells, one of which is the newly identified circRNA hsa_circ_0003511, here referred to as circLMBR1. We found that circLMBR1 expression was down-regulated in BC cells, suggesting its potential as a biomarker and a target for treatment. To explore its role in BC, we overexpressed and knocked down circLMBR1 in subsequent experiments. Through a series of *in vivo* and *in vitro* experiments, we found that circLMBR1 can inhibit the proliferation, cloning, migration, and invasion of BC cells, indicating that it is a tumor suppressor gene.

We then focused on the mechanism by which circLMBR1 plays an

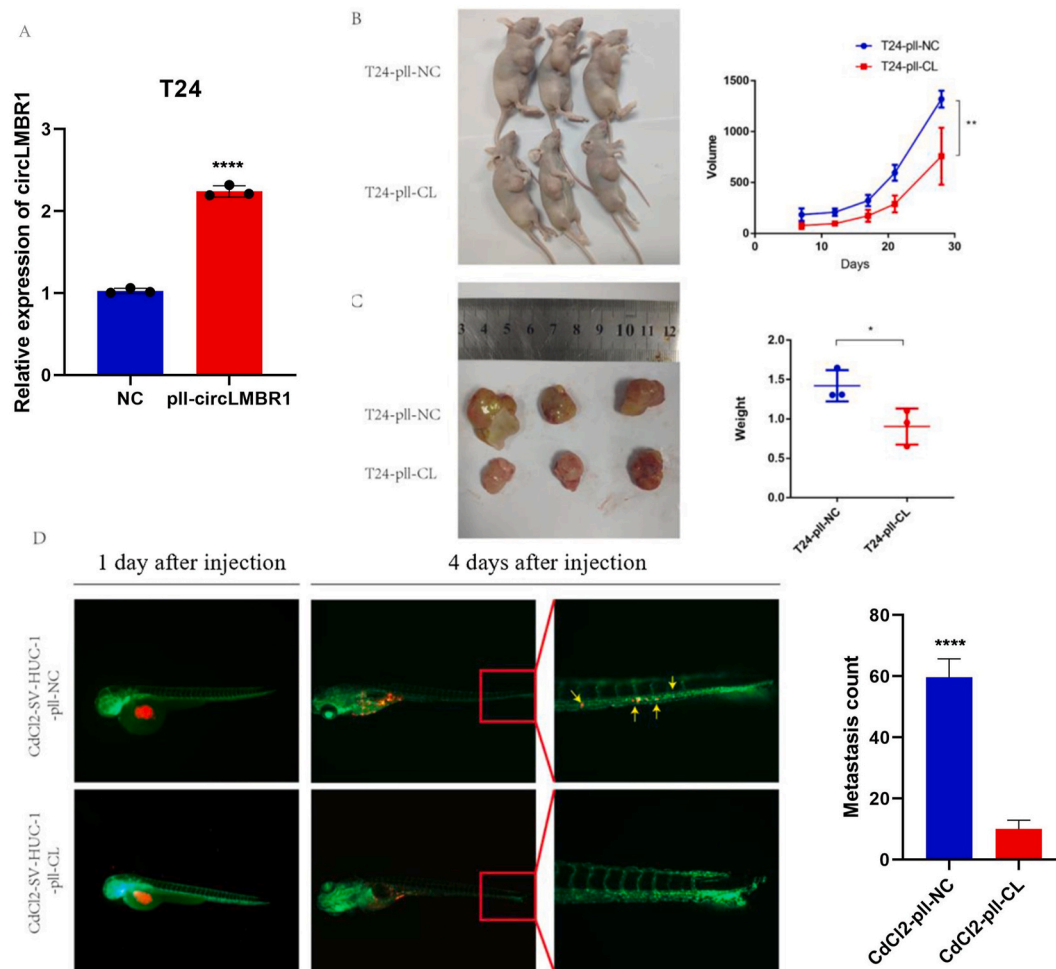


Fig. 5. Overexpression of circLMBR1 inhibits subcutaneous growth and metastasis of cells. **A.** Verification of circLMBR1 overexpression efficiency by qRT-PCR. **B.** Growth of subcutaneous tumors in nude mice, $n = 3$. **C.** Comparison of the weight of subcutaneous tumors in nude mice after 4 weeks of subcutaneous tumor formation, $n = 3$. **D.** Statistics of the location and tail metastasis of CdCl2-SV-HUC-1 cells overexpressing circLMBR1 into the yolk sac of zebrafish and the number of metastatic tail cells. Red is the cell, green is the zebrafish vascular endothelial cell, and the yellow arrow is the cell transferred to the zebrafish tail. $n = 50$; $*P < 0.05$, $**P < 0.01$. The figure shows that overexpression of circLMBR1 inhibits subcutaneous tumor growth and metastasis in mice and zebrafish respectively (A–D). Statistical analysis of the results demonstrates a significant decrease in tumor growth and metastasis ($**P < 0.01$).

inhibitory role in cancer. Recent reports have mainly focused on circRNA acting as a sponge of miRNA to regulate the expression of downstream genes [37]. For example, circACVR2A can inhibit the progression of BC by regulating the expression of EYA4 through sponged miR-626 [38]. Circ_0008532, meanwhile, sponges miR-155-5p and miR-330-5p and regulates the expression of their target gene MTGR1 to promote the progression of BC [39]. However, the regulatory role of circRNA and RNA binding proteins in BC has yet to be elucidated. Our research demonstrates that circLMBR1 can combine protein ALDH1A3 to inhibit the progression of BC (Fig. 9). Specifically, circLMBR1 up-regulates the expression of protein ALDH1A3 to inhibit the proliferation, cloning, invasion, and migration of BC cells *in vivo* and *in vitro*. This novel insight into the mechanism of circRNA and RNA binding proteins may provide new biomarkers for the diagnosis and treatment of BC.

The ALDH1A3 enzyme is a member of the aldehyde dehydrogenase (ALDH) superfamily and is known to irreversibly oxidize aldehydes to their corresponding acids [40,41]. It has been implicated in cancer regulation and is abnormally expressed in various cancer types, including pancreatic, ovarian, prostate, and non-small cell lung cancer [42–45]. In non-muscular invasive BC, ALDH1A3 promoter hypermethylation has been linked to decreased expression [46]. However, no studies have yet to be conducted on muscular invasive BC.

Our research presented novel evidence of circLMBR1 binding to ALDH1A3, with overexpression of circLMBR1 increasing the expression of ALDH1A3 and silencing ALDH1A3 reversing the anti-tumor effect of circLMBR1 and promoting BC cell proliferation, invasion, and migration. Although our findings have shed light on the regulatory role of circRNA in cancer progression, the specific mechanism of circLMBR1 in regulating ALDH1A3 expression remains to be elucidated.

Moreover, to date, no studies have investigated the changes of circRNA expression following chemical induction in BC cells. Most studies have focused solely on the role of circRNA in the development of BC without categorizing the source of BC cells [47]. In this study, we used high-throughput sequencing to identify the presence of the tumor suppressor circLMBR1 in chemically induced BC cells. We then confirmed the interaction between circLMBR1 and ALDH1A3 using RNA pulldown assay and mass spectrometry, demonstrating that circRNA is involved in the regulation of RNA binding protein expression, contrasting previous findings of circRNA encoding proteins [48]. Our findings provide promising new avenues for further research into the role of circRNA in tumor progression.

Our study provides compelling evidence that circLMBR1 can directly modulate ALDH1A3 expression to suppress BC cell growth and invasion. This is the first report to highlight the tumor suppressor role of circLMBR1 in BC, and provides an important foundational basis for

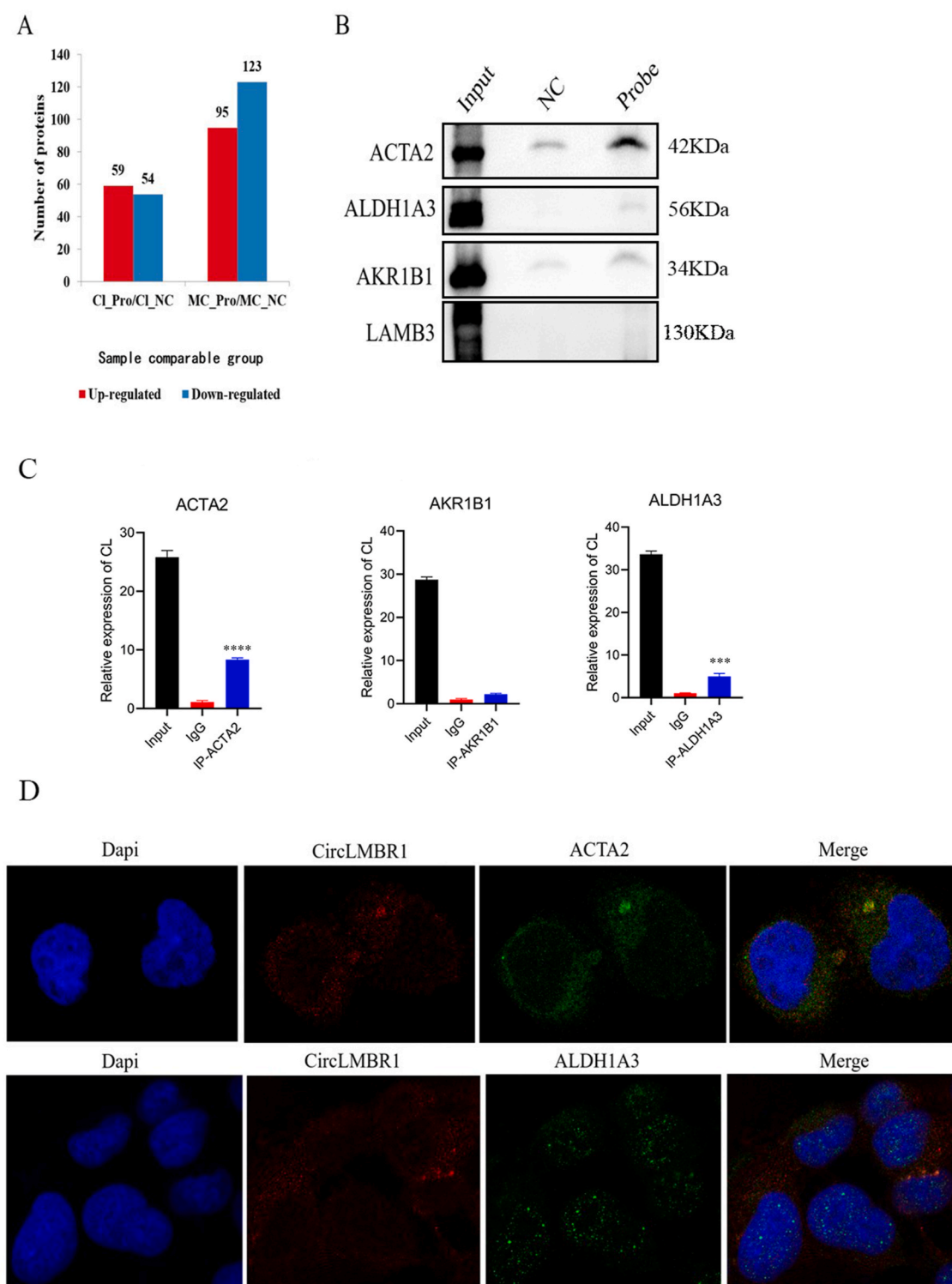


Fig. 6. CircLMBR1 binding with ACTA2 and ALDH1A3. **A.** Number of differential genes in Cdc12-SV-HUC-1 and MC-SV-HUC-1 cells. **B.** Western blot analysis of the eluent after RNA pulldown assay, showing ACTA2, ALDH1A3, and AKR1B1 being successfully pulled down by the probe. **C.** Reverse verification by qRT-PCR assay with the eluent after RIP assay. **D.** FISH assay of circLMBR1 in the cytoplasm, showing co-localization with ACTA2 and ALDH1A3.

further elucidating the molecular mechanisms of circRNA in BC development. Although further research is needed to elucidate the precise mechanism of circLMBR1 in regulating ALDH1A3 expression, our findings are an important step towards the development of novel therapeutic strategies for BC.

5. Conclusion

The RNA expression profile of malignant transformed bladder epithelial cells induced by chemicals has undergone a large number of changes, and we have identified many new circular RNA. Among them, the expression of circLMBR1 is down-regulated in bladder cancer cell lines. A series of experiments *in vivo* and *in vitro* have confirmed that

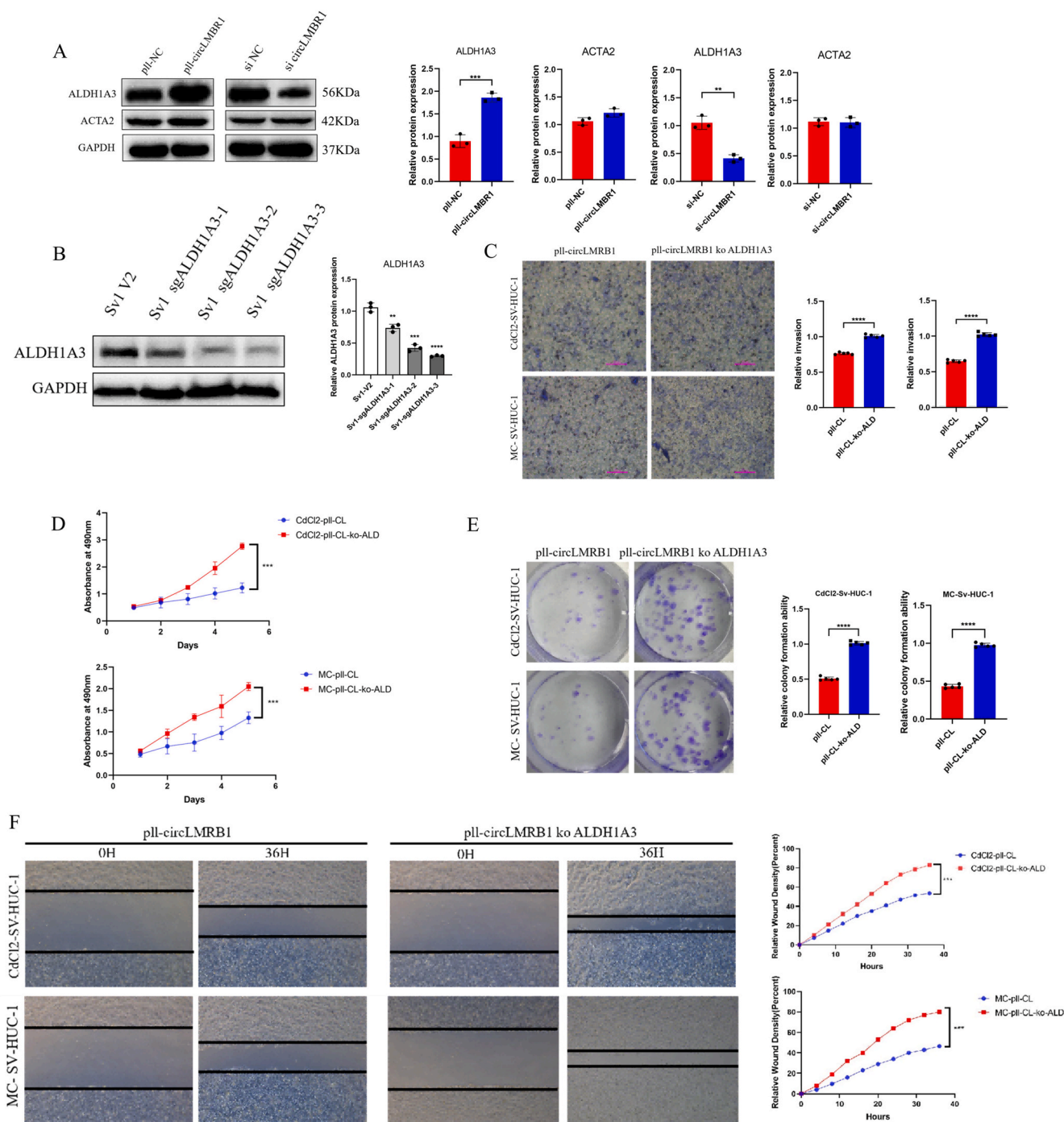


Fig. 7. Effects of ALDH1A3 on the inhibitory function of circLMBR1 on invasion, proliferation, cloning and migration of bladder malignant transformed cells. **A.** Overexpression of circLMBR1 up-regulated the expression of ALDH1A3 protein. **B.** Western blot analysis of ALDH1A3 expression in ALDH1A3 stable knockout cells. **C–F.** Transwell, proliferation, colony formation, and wound healing assays showed that knocking down the expression of ALDH1A3 reversed the inhibitory effect of circLMBR1 on invasion, proliferation, cloning and migration of malignant transformed cells.

circLMBR1 can inhibit the progression of bladder cancer cells. Furthermore, circLMBR1 can bind to the protein ALDH1A3 and up-regulate its expression to inhibit the progression of bladder cancer cells. In summary, circLMBR1 has the potential to be a target for diagnosis and treatment of bladder cancer.

Funding statement

This work was supported by the Guangzhou Municipal Science and Technology Bureau (201804020023 and SL2023A03J00557) and

Science and Technology Department of Xinjiang Uyghur Autonomous Region (2021D01A31).

Ethics approval and consent participate

All animal assays were approved by the Ethics Committee of Guangzhou Medical University (2018-192). Informed consent was obtained from the patients enrolled in this study. The study was approved by the Ethics Committee of Guangzhou Medical University with the approval number of GMU-IRB#: 2015–11.

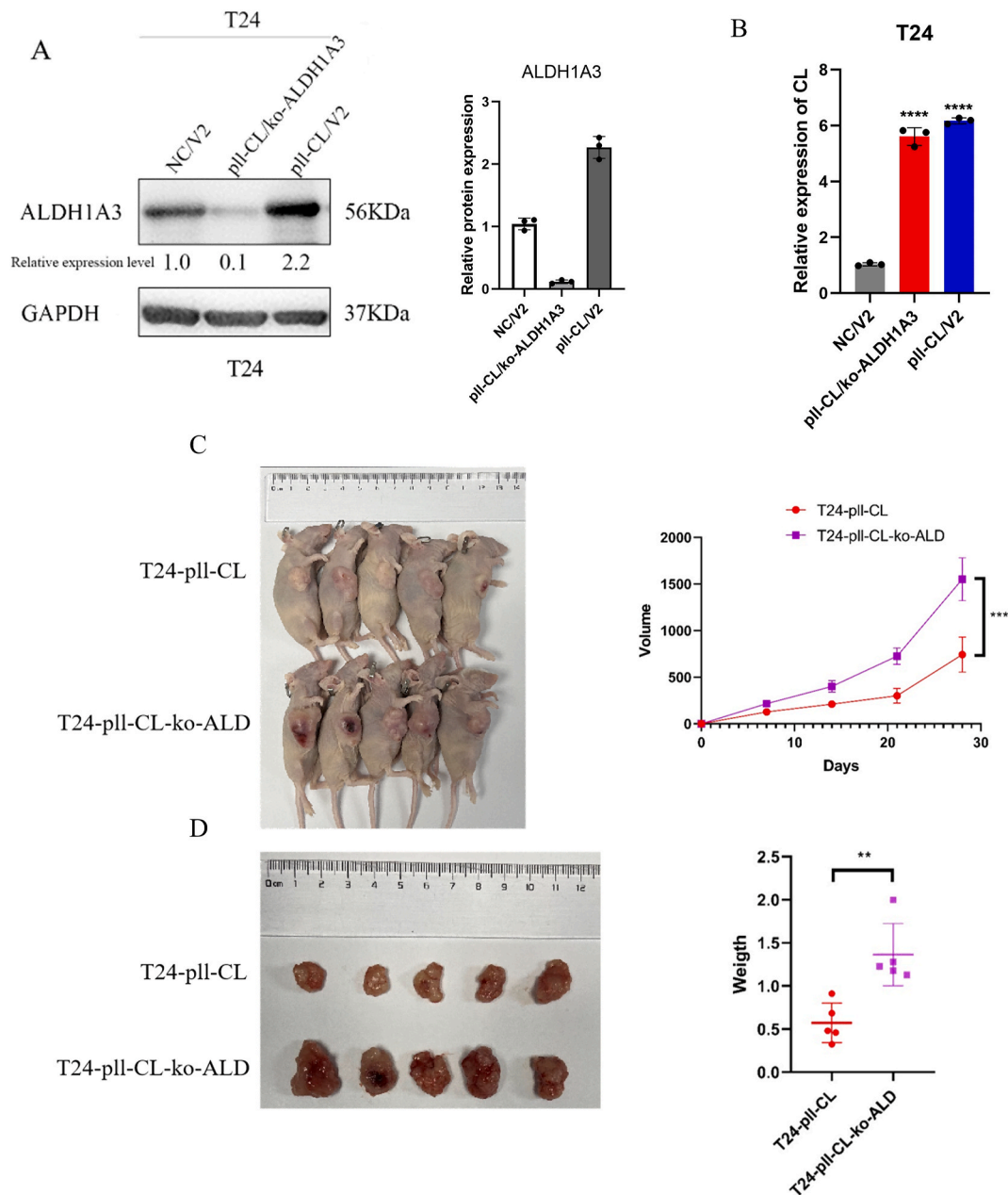


Fig. 8. Effects of knocking down ALDH1A3 and overexpressing circLMBR1 on subcutaneous tumor growth and metastasis. **A.** Verification of ALDH1A3 expression by Western blot. **B.** Verification of circLMBR1 overexpression efficiency by qRT-PCR. **C.** Growth of subcutaneous tumors in nude mice, n = 5. **D.** Comparison of the weight of subcutaneous tumors in nude mice after 4 weeks of subcutaneous tumor formation, n = 5; **P < 0.01, ***P < 0.001. This figure demonstrates that knocking down the expression of ALDH1A3 can reverse the function of overexpressed circLMBR1 in inhibiting subcutaneous growth of cells.

Consent to publish

All authors agree to publish this article.

CRedit authorship contribution statement

Yifan Lv: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Zusen Yuan:** Writing – review & editing, Writing – original draft, Validation, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Dongmao Chen:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Zhibin Chen:** Writing – original draft, Project administration,

Methodology, Investigation, Data curation. **Xiaowei Zhu:** Writing – original draft, Methodology. **Xiaoling Ying:** Writing – review & editing, Writing – original draft. **Yapeng Huang:** Writing – review & editing, Writing – original draft, Conceptualization. **Weidong Ji:** Writing – review & editing, Validation, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Defeng Qi:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors have no relevant financial or non-financial interests to disclose.

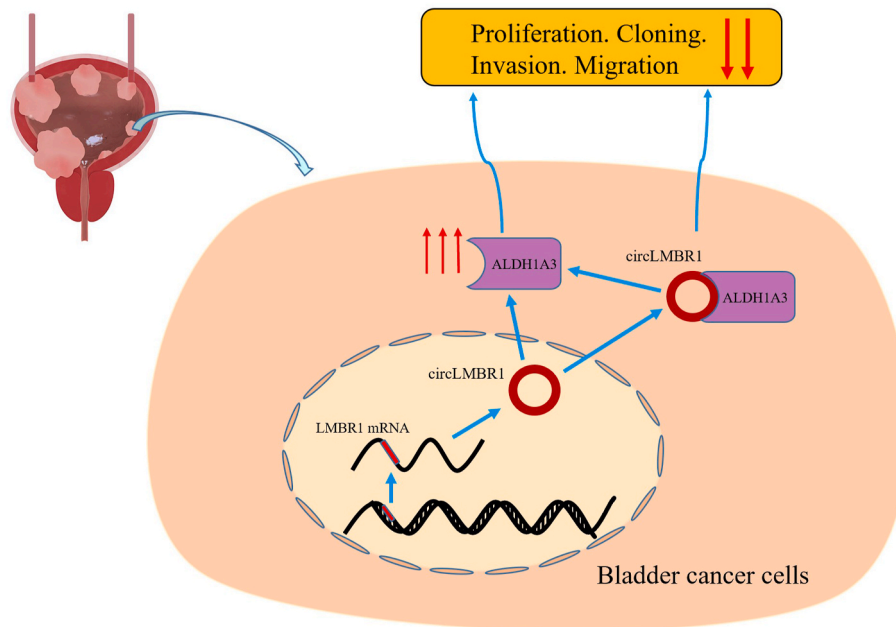


Fig. 9. Schematic of the action of circLMBR1 regulating bladder cancer progression. circLMBR1 binds to protein ALDH1A3 and enhancing its expression, thereby inhibits the invasion, proliferation, cloning and migration of bladder cancer.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ncrna.2024.05.004>.

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