



## ORIGINAL ARTICLE

# Hsa\_circ\_0003945 promotes progression of hepatocellular carcinoma by mediating miR-34c-5p/LGR4/ $\beta$ -catenin axis activity

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## Abstract

Accumulating evidence suggests that circular RNAs (circRNAs) play essential roles in regulating cancer progression, but many circRNAs in hepatocellular carcinoma (HCC) remain unknown. Dysregulated circRNAs in HCC were identified through bioinformatics analysis of Gene Expression Omnibus data sets. Quantitative real-time PCR (qRT-PCR), Sanger sequencing, RNase R digestion and actinomycin D treatment were conducted to confirm the characterization of circRNAs. CCK-8, wound-healing and Transwell assays were performed to assess the functional roles of Hsa\_circ\_0003945 (Circ\_0003945) in HCC cell lines. Subcellular fractionation and fluorescence in situ hybridization (FISH) were performed to locate Circ\_0003945 in HCC cells. Dual-luciferase reporter assay was executed to verify the binding of Circ\_0003945 to microRNAs (miRNAs) or the miRNAs to their target genes. In this study, we found that Circ\_0003945 was upregulated in HCC tissue, and higher Circ\_0003945 expression was positively correlated with tumour size and tumour stage. Furthermore, high plasma levels of circulating Circ\_0003945 were confirmed in HCC patients compared with those in non-HCC groups. The functional experiments revealed that overexpression or knockdown of Circ\_0003945 promoted or attenuated tumour growth and migration, respectively. Mechanistically, Circ\_0003945 might exert as a miR-34c-5p sponge to upregulate the expression of leucine-rich repeat-containing G

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protein-coupled receptor 4 (LGR4), activating the  $\beta$ -catenin pathway, and finally facilitating HCC progression. Additionally, a  $\beta$ -catenin activator could reverse the effect of Circ\_0003945 knockdown. In conclusion, Circ\_0003945 exerts a tumour-promoting role in HCC cells by regulating the miR-34c-5p/LGR4/ $\beta$ -catenin axis, which may be a potential target for HCC therapy.

#### KEYWORDS

Circ\_0003945, hepatocellular carcinoma, leucine-rich repeat-containing G protein-coupled receptor 4, miR-34c-5p, tumour progression

## 1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumours globally.<sup>1</sup> Surgery remains the most effective treatment strategy, with curative potential in well-selected candidates.<sup>2</sup> Despite improved surveillance and treatment strategies in recent years, the clinical outcome of HCC remains dismal due to the high rates of relapse and metastasis, which result in a poor 5-year survival rate.<sup>3,4</sup> Thus, a thorough investigation into the mechanisms underlying HCC progression and metastasis is urgently needed to develop new therapeutic approaches and improve the clinical outcome of HCC patients.

CircRNAs are generated via proactive back-splicing of pre-mRNA and are endowed with neither 5' to 3' polarity nor a polyadenylated tail, but they are characterized by covalently closed-loop structures and show resistance to ribonuclease R (RNase R) digestion.<sup>5-7</sup> Accumulating evidence indicates that circRNAs are abnormally expressed in many types of cancers and are closely related to cancer progression and prognosis.<sup>8-10</sup> Furthermore, circRNAs might mediate pathological processes of cancer cells,<sup>11,12</sup> such as acting as a miRNA sponge to mediate the biological function of their downstream targets or regulate their parental gene expression.<sup>9,13</sup> Although some aberrant circRNAs play important roles in HCC tumorigenesis and progression,<sup>14-16</sup> the overall biological and molecular contributions of most circRNAs to HCC progress remain elusive.

In the present study, dysregulated circRNAs in HCC were screened by analysing Gene Expression Omnibus (GEO) datasets followed by verification in clinical samples. We further characterized one significantly overexpressed circRNA derived from exons 11 and 12 of the ubiquitin-associated protein 2 (UBAP2) and named Hsa\_circ\_0003945 (Circ\_0003945) in the circBase database. The function and mechanism of Circ\_0003945 in HCC progression were also investigated.

## 2 | MATERIALS AND METHODS

### 2.1 | Methods for screening circRNA profiles of HCC tissue

To show the dysregulated circRNAs, GEO databases (<https://www.ncbi.nlm.nih.gov/geo/>; GSE78520, GSE94508 and GSE97332) were selected based on inclusion of results of circRNA microarray

analysis of HCC tissue and paired healthy adjacent liver tissue.<sup>17,18</sup> The GEO2R tool was used to screen the dysregulated circRNAs in HCC tissue, with a  $p$  value  $<0.05$  used as a cut-off threshold.

### 2.2 | Clinical specimens

All human HCC tissue and paired adjacent normal liver tissue ( $n = 50$ ) and plasma samples ( $n = 151$ , including 41 cases of healthy donors [HD], 40 cases of chronic liver hepatitis [CHB], 30 cases of liver cirrhosis [LC] and 40 cases of HCC) were collected from the Zhongshan Hospital of Fudan University from September 2018 to December 2020 and stored at  $-80^{\circ}\text{C}$  until analysis. The criteria for diagnosing CHB and LC were consistent with our previous work.<sup>19</sup> The final diagnosis of HCC was verified histologically by two pathologists. The present study was approved by the Medical Ethics Committee of Zhongshan Hospital, Fudan University. All participants in this study provided written informed consent in accordance with the Declaration of Helsinki.

### 2.3 | Cell lines and cell culture

LO2 normal liver cells, HepG2 hepatoma cells and HCC cell line, MHCC97L, MHCC97H and HCCLM3 were obtained from the Liver Cancer Institute, Fudan University (Shanghai, China). SUN387, SK-Hep1, Li-7, Hep3B and human embryonic kidney (HEK-293T) cell lines were purchased from the cell bank at the Institute of Biochemistry and Cell Biology, China Academy of Science (Shanghai, China). All cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM; Cat. 10566016, Gibco) supplemented with 10% foetal bovine serum (FBS, Cat. BS-0003-500, BioSUN), 1% penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. Cells were cultured in a humidified atmosphere of 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ . Further experimental methods *in vitro* were shown in supplementary materials and methods.

### 2.4 | Animal experiments

Male BALB/c nude mice (5–6 weeks old) were purchased from the Department of Experimental Animals of the Chinese Academy

of Sciences (Shanghai, China). HCCLM3 cells transfected with shCirc\_0003945 or shNC ( $5 \times 10^6$  cells/mouse) were injected subcutaneously into the right dorsum to generate subcutaneous tumours ( $n = 6/\text{group}$ ). Mouse body weight and tumour size were measured every week. Four weeks later, mice were sacrificed and tumours were weighed and processed for histological analysis. Tumour volume was calculated according to the formula: volume ( $\text{mm}^3$ ) = width<sup>2</sup> × length/2. All animal care and procedures were performed following guidelines approved by the Institutional Animal Care and Use Committee at Zhongshan Hospital, Fudan University.

## 2.5 | Statistical analysis

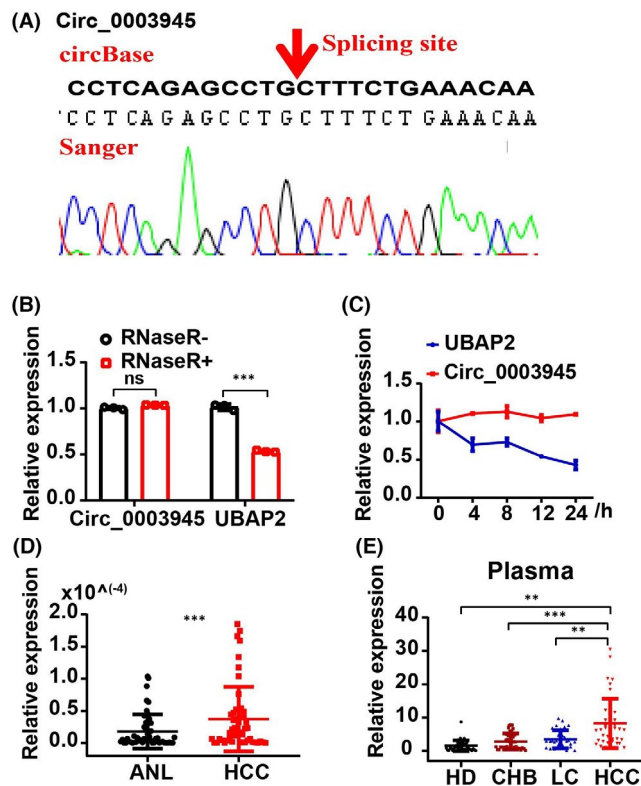
Statistical analysis was performed using Statistical Program for Social Sciences (SPSS) 24.0 Software (SPSS) and GraphPad Prism 8.0 (GraphPad Software). The distribution of each group was determined by the Kolmogorov-Smirnov test. Student's *t*-test (two-tailed) was used to assess statistical significance between the two groups. The paired *t*-test was used to analyse the statistical significance of circRNAs, miRNAs, or mRNAs between HCC and adjacent normal liver tissue. Chi-square test was used to analyse the correlation between circRNA level and clinicopathological features of HCC patients. Pearson's correlation or Spearman correlation was used to assess the correlation between circRNA and miRNA or mRNA. All experiments were repeated at least three times. Data are presented as mean ± SD.  $p < 0.05$  was considered statistically significant.

## 3 | RESULTS

### 3.1 | Characteristics and expression of Circ\_0003945 in HCC tissue and cell lines

To screen dysregulated circRNA expression profiles in HCC tissue, we re-analysed the datasets from the GEO database (Figure S1A). Ten significant candidate circRNAs were selected for further validation according to the  $p$  value ( $p < 0.05$ ),  $|\text{Log}_2\text{FC}| > 1.5$  (Table S1). Finally, Hsa\_circ\_0001955 (Circ\_0001955), Hsa\_circ\_0005397 (Circ\_0005397), Hsa\_circ\_0027478 (Circ\_0027478) and Hsa\_Circ\_0003945 (Circ\_0003945) were successfully verified. The back-spliced regions of these circRNAs were confirmed by Sanger sequencing, and all were in agreement with circBase (Figure and Figure S1B). Furthermore, RNase R digestion revealed that candidate circRNAs were more resistant than associated mRNAs (Figure 1B and Figure S1C), and actinomycin D treatment clearly attenuated the half-life of parental mRNA levels in HCC cells, but had little effect on circRNAs (Figure 1C and Figure S1D). These results confirmed the circular structure of circRNAs, showing that circRNAs were more stable than their linear forms.

Then, the expression level of circRNAs was measured by qPCR in HCC samples. We found that Circ\_0001955, Circ\_0005397 and Circ\_0003945 were significantly increased in HCC tissue compared with adjacent normal liver tissue (Figure 1D and Figure S2A-2B),



**FIGURE 1** Characteristics and expression of Circ\_0003945 in HCC samples. (A) Sanger sequencing of the qPCR product. Red arrow indicates the back-splicing sites of Circ\_0003945. (B) The qPCR analysis of Circ\_0003945 and associated UBAP2 in HCC cells treated with or without RNase R. (C) Expression levels of Circ\_0003945 and UBAP2 mRNA was determined in HCC cells treated with actinomycin D at indicated time points. (D) Expression level of Circ\_0003945 was analysed by qPCR in adjacent neighbouring liver (ANL) and HCC tissue; paired *t*-test was used. (E) Relative expression level of circulating Circ\_0003945 in plasma; non-parametric test was used. The RNase R treatment and actinomycin D experiments were performed in triplicate and *t*-tests were used. (\*\* $p < 0.01$ ; \*\*\* $p < 0.001$ )

with Circ\_0003945 showing the most significant increase of the differentially expressed circRNAs ( $p < 0.001$ , Figure 1D); no significant difference was found in the transcription of Circ\_0027478 (Figure S2C). Further analysis showed that higher expression level of Circ\_0003945 in HCC tissue was closely associated with tumour size and China liver cancer staging (CNLC) stage, implying a possible role of Circ\_0003945 in HCC progression (Table 1). Moreover, circulating Circ\_0003945 in plasma was also significantly increased in HCC patients compared with non-HCC groups (HD, CHB and LC; Figure 1E), indicating its potential diagnostic role.

### 3.2 | Circ\_0003945 promotes the proliferation and migration of HCC cells *in vitro*

To investigate the functional role of Circ\_0003945 in HCC, the Circ\_0003945 expression level in HCC cell lines was measured and

**TABLE 1** Correlations between Circ\_0003945 expression level and clinicopathological characteristics in HCC

Clinicopathological characteristics	Circ_0003945		p value
	Low	High	
Age			0.156
<60	9	14	
≥60	16	11	
Gender			0.462
Male	19	22	
Female	6	3	
Liver cirrhosis			0.777
Yes	12	13	
No	13	12	
Tumour number			0.247
Solidarity	19	23	
Multiple	6	2	
Tumour Size (cm)			0.009
≤5	14	5	
>5	11	20	
Tumour encapsulation			0.771
Complete	10	9	
None	15	16	
Tumour differentiation			0.156
I-II	14	9	
III-IV	11	16	
Microvascular invasion			0.544
No	7	9	
Yes	18	16	
CNLC			0.023
I	15	7	
II+III	10	18	
AFP (ng/ml)			0.556
<20	10	8	
≥20	15	17	

Note: CNLC, China liver cancer staging; AFP, alpha fetoprotein.

showed that Circ\_0003945 was highly expressed in most HCC cell lines compared with normal liver cell line (LO2; Figure 2A). Next, a specific shRNA targeting Circ\_0003945 was designed and transfected into HCCLM3 and MHCC97H cells, and a Circ\_0003945 overexpression vector was constructed and transfected into Li-7 cells. Circ\_0003945 expression was significantly decreased in HCCLM3 and MHCC97H cells (Figure 2B) and did not affect the level of its parental gene (UBAP2) at the mRNA and protein levels (Figure S3A,B). Dramatic upregulation of Circ\_0003945 was found in overexpression groups (Figure 2C), with UBAP2 expression unaffected (Figure S3C-3D).

Next, functional analysis showed that downregulation of Circ\_0003945 attenuated viability of HCC cells (Figure 2D), whereas overexpression of Circ\_0003945 increased viability of Li-7 cells (Figure 2E). Wound-healing assays implied that Circ\_0003945 downregulation significantly decreased the migratory ability of HCC cells (Figure 2F), whereas Circ\_0003945 overexpression revealed an opposite role (Figure 2G). Transwell assays showed a similar effect of Circ\_0003945 in HCC cell lines (Figure 2H-I).

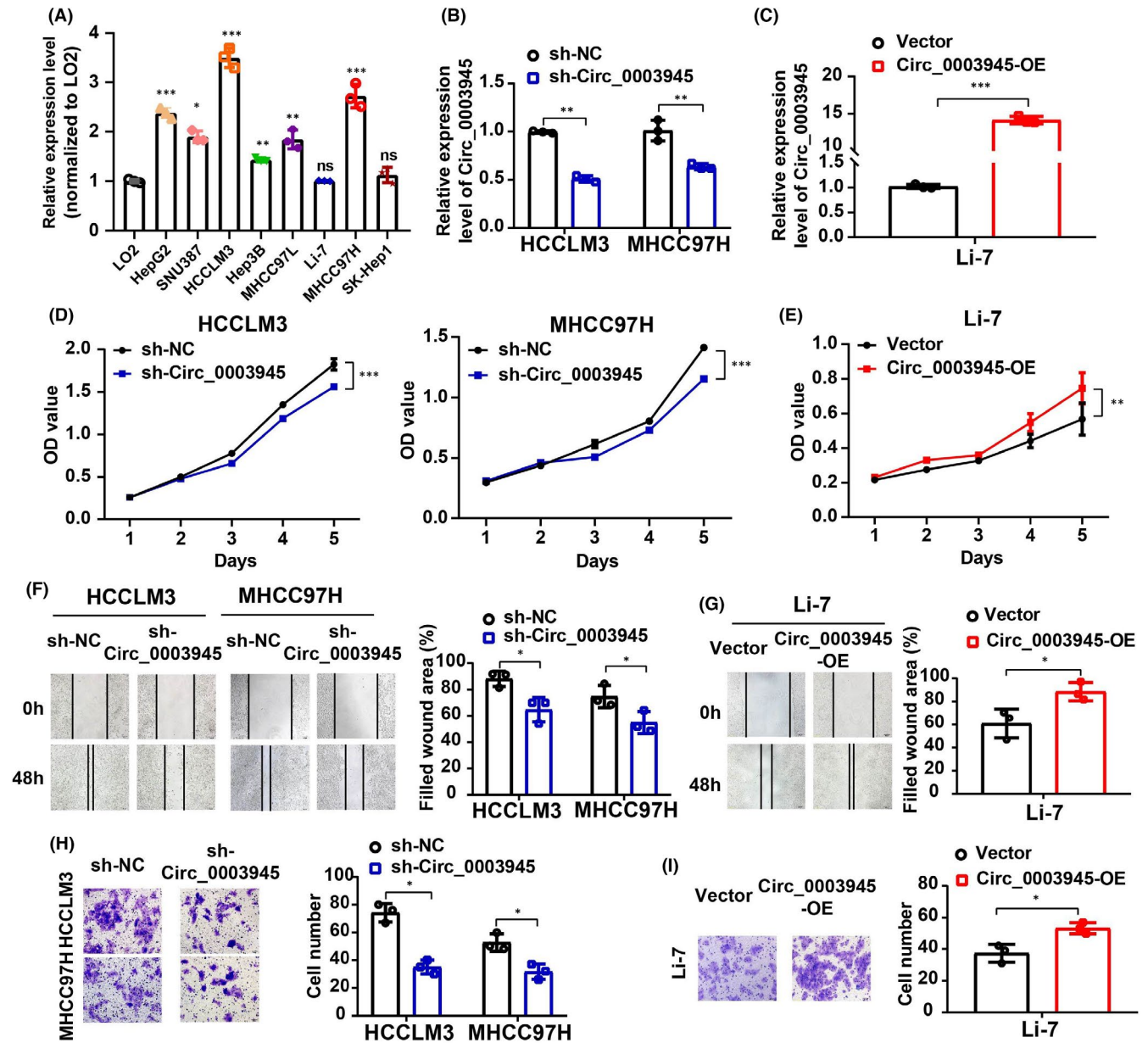
### 3.3 | Circ\_0003945 acts as a miR-34c-5p sponge in HCC cells

To explore the underlying mechanism of Circ\_0003945 in HCC progression, subcellular fractionation analysis of Circ\_0003945 was first performed to determine whether circRNAs play different roles depending on their localization in cells.<sup>5,20</sup> This analysis revealed that Circ\_0003945 was mostly located in the cytoplasm of HCC cells (Figure 3A). Further FISH analysis also verified this (Figure 3B), implying that Circ\_0003945 may act as a miRNA sponge to participate in HCC progression.

The regulatory effects of miRNAs on their target genes are dependent on RNA-induced silencing complex (RISC), which contains the protein AGO2.<sup>21,22</sup> Therefore, RIP analysis was conducted using anti-AGO2 antibody in HCC cells. We found that the anti-AGO2 antibody significantly bound Circ\_0003945, but not circANRIL (a circular RNA that does not bind AGO2 and was used as a negative control<sup>23,24</sup>), indicating that Circ\_0003945 might serve as a platform for AGO2 and miRNAs (Figure 3C).

Subsequently, the putative miRNA targets were predicted using three publicly available prediction tools (CircInteractome, circBank and miRanda). Two miRNAs (hsa-miR-34c-5p [miR-34c-5p]) and hsa-miR-571 [miR-571]) were selected as possible targets of Circ\_0003945 for their high comprehensive score (Figure S4A; Table S2). A further dual-luciferase reporter assay was performed to investigate whether miR-34c-5p or miR-571 targets Circ\_0003945 (Figure S4B). MiR-34c-5p mimics or miR-571 mimics were co-transfected with the luciferase reporters (which contained wild-type or mutant miR-34c-5p/miR-571 target sequences of Circ\_0003945) into HEK-293T cells. Compared with the negative control miRNA (miR-NC), miR-34c-5p significantly decreased luciferase reporter activity in the cells compared with the wild-type Circ\_0003945 sequence, but not in mutant binding groups (Figure 3D). However, no significant effect was found in a similar experimental system where cells were transfected with miR-571 mimics (Figure 3E). Therefore, Circ\_0003945 might be a miR-34c-5p sponge.

Next, the association between Circ\_0003945 and miR-34c-5p was addressed. The miR-34c-5p was expressed at a lower level in HCC tissue than in adjacent normal tissue (Figure 3F), and a negative correlation was found between Circ\_0003945 and miR-34c-5p in tissue samples (Figure 3G). Further, knockdown



**FIGURE 2** Effects of Circ\_0003945 on proliferation, migration and invasion of HCC cells. (A) Relative expression level of Circ\_0003945 in LO2 normal hepatocytes and HCC cell lines. (B and C) Expression of Circ\_0003945 in stably transfected HCC cells. (D and E) Effect of Circ\_0003945 knockdown or overexpression on the proliferation of HCC cells was monitored via CCK-8 assays. (F and G) Migratory ability was assessed by wound-healing assays in Circ\_0003945-knockdown or overexpressing HCC cells. Scale bar = 100  $\mu$ m. (H and I) Migratory ability was measured by Transwell assay in Circ\_0003945-knockdown or overexpressing HCC cells. Scale bar = 50  $\mu$ m. All in vitro experiments were performed in triplicate and t-tests were used. (\* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001, ns, not significant)

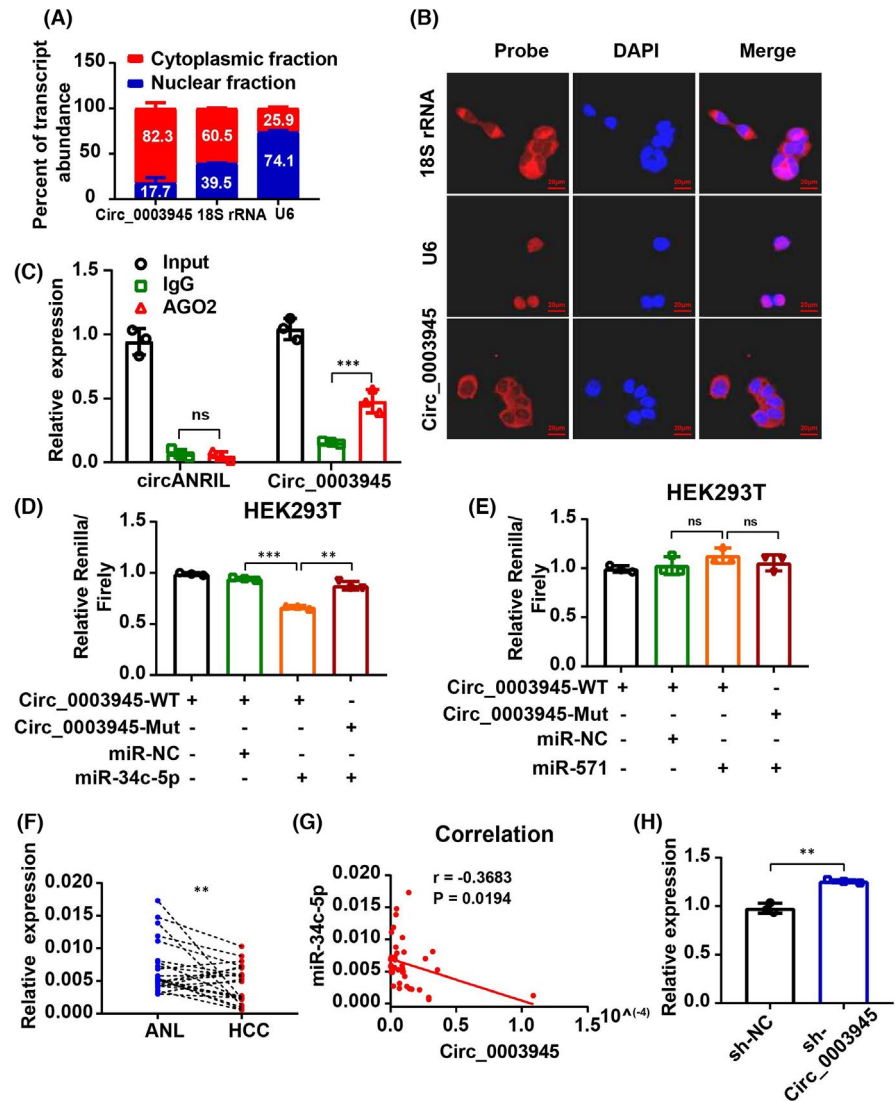
of Circ\_0003945 upregulated miR-34c-5p expression level (Figure 3H).

### 3.4 | Transfection of Circ\_0003945 antagonizes the inhibitory effects of miR-34c-5p on HCC progression *in vitro*

Next, functional analysis was carried out to evaluate the effect of Circ\_0003945 sponging miR-34c-5p. Results showed that inhibiting

miR-34c-5p expression reversed the reduced cell viability induced by Circ\_0003945 knockdown (Figure S4C; Figure 4A), and the miR-34c-5p mimics attenuated the promotional effects of Circ\_0003945 overexpression on proliferation (Figure S4D; Figure 4B). By analogy, suppressing miR-34c-5p expression enhanced the role of Circ\_0003945 in maintaining the migratory ability of HCC cells (Figure 4C). Meanwhile, miR-34c-5p mimics dramatically inhibited the migration of HCC cells induced by Circ\_0003945 overexpression (Figure 4D). A similar effect was observed in Transwell assays (Figure 4E-F). These results collectively illustrate that Circ\_0003945

**FIGURE 3** Circ\_0003945 acts as miR-34c-5p sponge in HCC cells. (A) The location of Circ\_0003945 was confirmed by subcellular fractionation. (B) FISH analysis was performed to observe the cellular location of Circ\_0003945, 18S rRNA and U6 in MHCC97H cells. Circ\_0003945, 18S rRNA and U6 probe were labelled with Cy3 (red) and nuclei were counterstained with DAPI (blue). Scale bar = 20  $\mu$ m. (C) RIP experiment was performed to co-immunoprecipitate AGO2 complex. (D and E) Relative luciferase activity in HEK-293T cells co-transfected with miR-34c-5p or miR-571 and Circ\_0003945-WT or Circ\_0003945-Mut vector. (F) Expression level of miR-34c-5p was determined by qPCR and paired t-test was used. (G) Correlation between miR-34c-5p and Circ\_0003945 in tissue samples was evaluated using Spearman correlation analysis. (H) Expression level of miR-34c-5p in Circ\_0003945-knockdown HCC cells. All in vitro experiments were performed in triplicate and t-test were used. (\* $p < 0.05$ ; \*\* $p < 0.01$ ; ns, not significant)



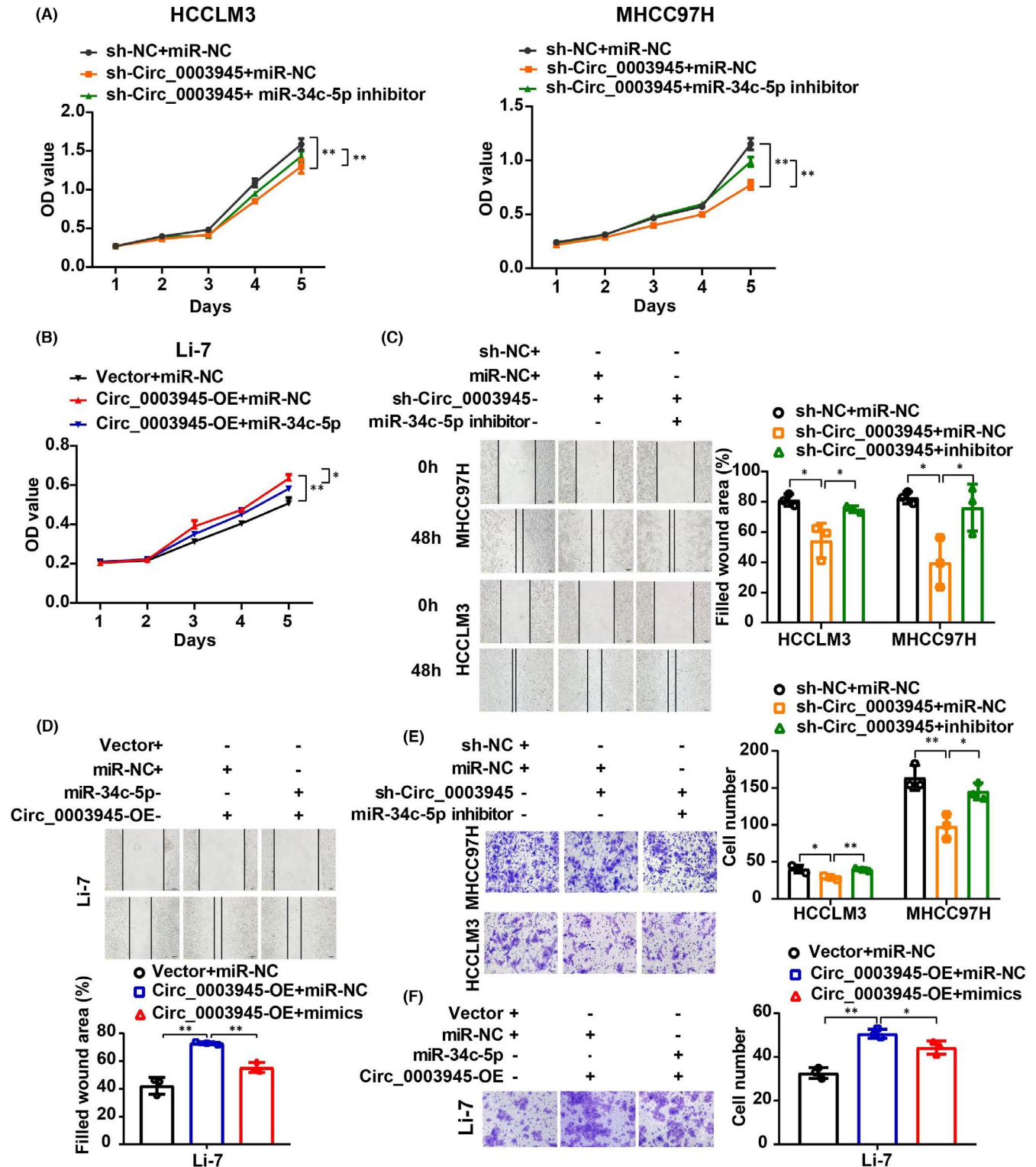
is necessary to maintain HCC cell progression in part by absorbing miR-34c-5p.

### 3.5 | Circ\_0003945 contributes to HCC progression by promoting LGR4 expression by sponging miR-34c-5p

The miRNAs have been reported to regulate their downstream target genes by recognizing the guide sequence. The downstream targets of miR-34c-5p were predicted according to the TargetScan and miRDB databases. Based on the comprehensive score, immunoglobulin superfamily member 1 (IGSF1), CUE domain-containing protein 1 (CUEDC1), cell death-inducing p53 target 1 (CDIP1), small G protein signalling modulator 2 (SGSM2), and leucine-rich repeat-containing G protein-coupled receptor 4 (LGR4) were finally selected for further verification (Table S3). The qPCR results showed that only LGR4 expression was dramatically decreased by transfection of miR-34c-5p mimics compared with miR-NC, and LGR4 was

upregulated by suppressing miR-34c-5p expression (Figure S5A). Western blot analysis also confirmed this (Figure S5B). Further, luciferase reporters were constructed to verify this interaction (Figure S5C). Luciferase assay showed that transfection of miR-34c-5p mimics dramatically decreased the activity of the luciferase reporter carrying the wild-type LGR4 3' untranslated region (3'-UTR) compared with the miR-NC group. In contrast, the mutated luciferase reporter showed no significant change with miR-34c-5p overexpression (Figure S5D). Additionally, LGR4 was expressed at remarkably high level in HCC tissue compared with adjacent normal liver specimens (Figure S5E). A negative correlation was also found between the level of miR-34c-5p and LGR4 expression in tissue samples (Figure S5F).

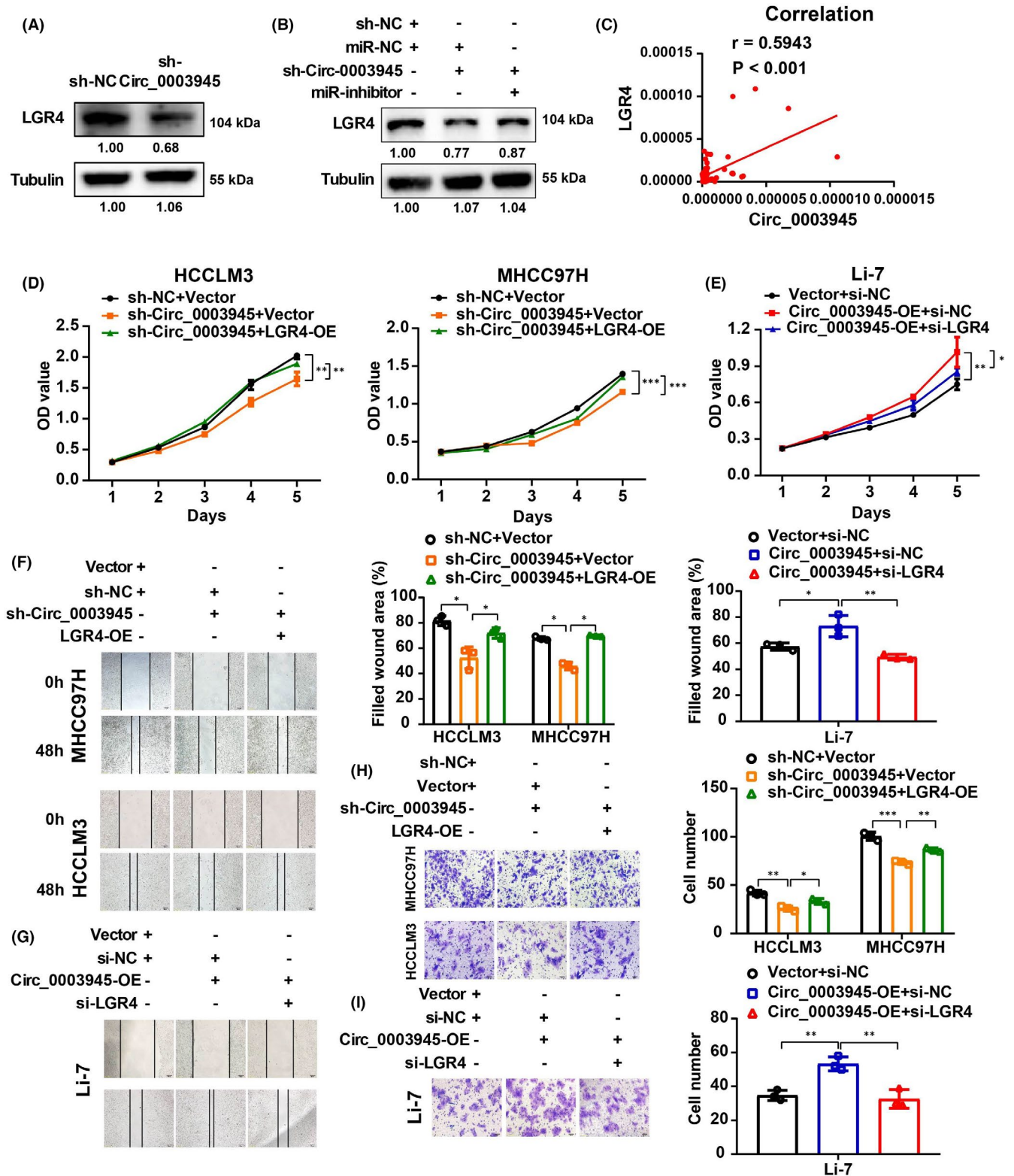
As Circ\_0003945 may function as a miR-34c-5p sponge to regulate LGR4 expression, the relationship between Circ\_0003945 and LGR4 was also addressed. Western blot analysis revealed that LGR4 expression significantly decreased with decreasing level of Circ\_0003945 (Figure 5A and Figure S5G), and the suppressed LGR4 level in Circ\_0003945-knockdown HCC cells was promoted



**FIGURE 4** Enhanced expression of Circ\_0003945 attenuates miR-34c-5p-mediated role of HCC progression *in vitro*. (A and B) Viability of HCC cells with changing expression of Circ\_0003945 or miR-34c-5p or both via CCK-8 assay. (C and D) Migratory ability of HCC cells with altering expression of Circ\_0003945 and miR-34c-5p assessed by wound-healing assay. Scale bar = 100  $\mu$ m. (E and F) The migratory ability of HCC cells with changing expression of Circ\_0003945 and miR-34c-5p was assessed by Transwell assay. Scale bar = 50  $\mu$ m. All *in vitro* experiments were performed in triplicate and t-tests were used. (\* $p$  < 0.05; \*\* $p$  < 0.01)

by miR-34c-5p inhibition (Figure 5B and Figure S5H). Clinically, we also found that Circ\_0003945 level was positively correlated with LGR4 expression (Figure 5C).

We further explored the biological role of LGR4 induced by Circ\_0003945. Functional analysis showed that repressed proliferation of Circ\_0003945-knockdown HCC cells was rescued by LGR4



**FIGURE 5** Circ\_0003945 contributes to HCC progression by regulating LGR4. (A) Western blot analysis of LGR4 in Circ\_0003945-knockdown HCC cells. (B) Level of LGR4 in HCC cells with Circ\_0003945-knockdown or miR-34c-5p alteration. (C) The correlation between LGR4 and Circ\_0003945 expression; Spearman's rank correlation test was used. (D and E) Proliferation assay of HCC cells with silencing or overexpressing Circ\_0003945 and LGR4. (F-I) Migration of HCC cells after silencing or overexpressing Circ\_0003945 and LGR4. For F and G, scale bar = 100  $\mu$ m, for H and I, scale bar = 50  $\mu$ m. Functional experiments were performed in triplicate and t-tests were used. (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ )



overexpression (Figure 5D). Meanwhile, enhanced proliferation of Circ\_0003945-overexpressing HCC cells was suppressed by suppressing LGR4 expression (Figure 5E). The migration assay revealed similar results (Figure 5F-5I).

### 3.6 | Circ\_0003945/miR-34c-5p/LGR4 promotes HCC progression via affecting $\beta$ -catenin pathway

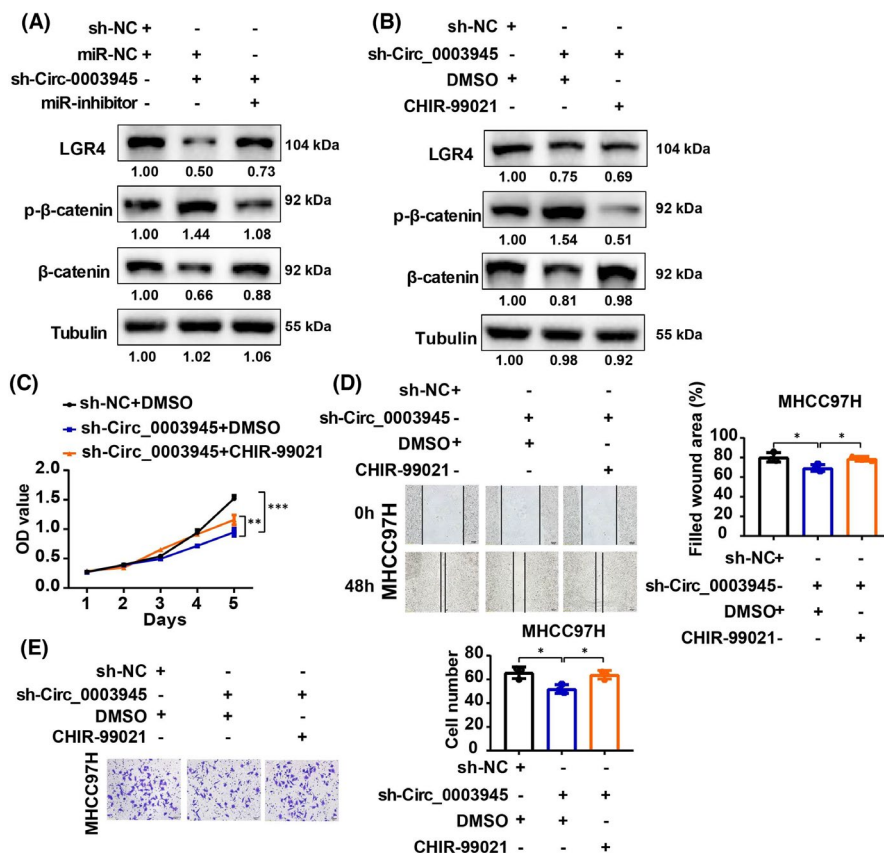
To identify the signalling pathway underlying the effects of the Circ\_0003945/miR-34c-5p/LGR4 axis on HCC cells, KEGG analysis (Entry:map 04310) and previous studies showed that LGR4 is the key gene in the Wnt/ $\beta$ -catenin pathway.<sup>25,26</sup> Therefore, we investigated whether  $\beta$ -catenin pathway was influenced by the Circ\_0003945/miR-34c-5p/LGR4 axis. It revealed that stable knockdown of Circ\_0003945 contribute to the phosphorylation of  $\beta$ -catenin and miR-34c-5p reversed this effect (Figure 6A and Figure S6A), which may induce accumulation of  $\beta$ -catenin. Nuclear and cytoplasmic protein assays also confirmed that the accumulation of  $\beta$ -catenin in the nucleus in overexpressing-Circ\_0003945 HCC cells (Figure S6B). The representative downstream target genes of the  $\beta$ -catenin pathway (c-Myc and Cyclin D1) were attenuated by silencing Circ\_0003945 (Figure S6C,D) and miR-34c-5p also participated in this process.

To further determine whether the  $\beta$ -catenin pathway is regulated by Circ\_0003945, CHIR-99021<sup>27</sup> (an activator of Wnt/ $\beta$ -catenin)

was used. We found that CHIR-99021 suppressed the phosphorylation of  $\beta$ -catenin in Circ\_0003945-knockdown MHCC97H cells (Figure 6B; Figure S6E). In addition, CCK-8 assay showed that CHIR-99021 reversed the reduced cell viability of Circ\_0003945 knockdown (Figure 6C). Transwell assays also indicated a similar role of CHIR-99021 in migration and invasion of HCC cells (Figure 6D,E). Moreover,  $\beta$ -catenin was knockdown in Circ\_0003945-overexpressing HCC cells, and we found that Circ\_0003945 enhanced cell proliferation and migration, while intervening  $\beta$ -catenin could inhibit the enhanced role of Circ\_0003945 (Figure S7A-C).

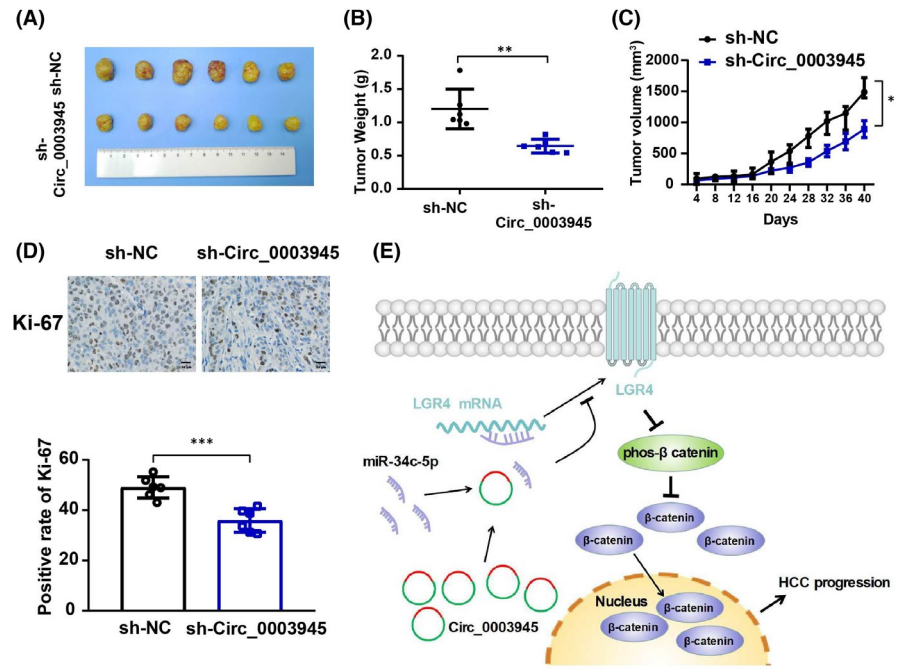
### 3.7 | Circ\_0003945 promotes the growth of HCC tumours *in vivo*

To validate the phenotype of Circ\_0003945 in HCC progression *in vivo*, we subcutaneously injected nude mice with Circ\_0003945-knockdown HCCLM3 cells. Circ\_0003945 knockdown significantly decreased tumour volume and tumour weight in nude mice (Figure 7A-C). Further, immunohistochemical analysis of tumours generated from HCC cells showed that the level of Ki-67 expression (an indicator of cell proliferation) was markedly decreased in Circ\_0003945-knockdown groups (Figure 7D). In conclusion, Circ\_0003945 acts as a miR-34c-5p sponge to upregulate LGR4, activating the  $\beta$ -catenin axis and promoting HCC progression (Figure 7E).



**FIGURE 6**  $\beta$ -Catenin pathway was finally activated by Circ\_0003945/miR-34c-5p/LGR4 to promote HCC progression. (A) Western blot analysis of the  $\beta$ -catenin pathway induced by the Circ\_0003945/miR-34c-5p axis in HCC cells. (B) The effect of  $\beta$ -catenin activator on Circ\_0003945-knockdown HCC cells. (C) Measurement of proliferation of HCC cells treated with  $\beta$ -catenin activator after silencing of Circ\_0003945 in HCC cells. (D-E) Migratory ability of decreasing-Circ\_0003945-HCC cells after treatment with  $\beta$ -catenin activator. For figure D, scale bar = 100  $\mu$ m. For figure E, scale bar = 50  $\mu$ m. All experiments were performed in triplicate and t-tests were used. (\* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001)

**FIGURE 7** Circ\_0003945 promotes the growth and metastasis of HCC cells *in vivo*. (A) Tumours were dissected from nude mice subcutaneously injected with Circ\_0003945-knockdown or control MHCC97H cells. (B and C) Weight and volume of subcutaneous xenograft tumours isolated from nude mice. (D) Representative images of immunohistochemical analysis to detect Ki-67. Scar bar = 50  $\mu$ m. (E) Schematic diagram indicating the underlying mechanism by which Circ\_0003945 enhances HCC progression via the miR-34c-5p/LGR4 axis. (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ )



## 4 | DISCUSSION

Although accumulating research has revealed that circRNAs clearly show abnormal expression related to tumour growth and progression in HCC,<sup>17,28-30</sup> the function of numerous circRNAs in HCC remains elusive and needs to be further investigated. In the current study, the expression of Circ\_0003945, originating from exon 11 and 12 of UBAP2, was significantly upregulated in HCC tissue and plasma. Further analysis indicated that higher expression of Circ\_0003945 was correlated with tumour size and tumour stage in HCC tissue. Functionally, Circ\_0003945 affected a series of properties of HCC cells, including tumour cell proliferation and migration. Mechanistically, Circ\_0003945 acted as a miR-34c-5p sponge to upregulate LGR4 expression, contributing to HCC progression by regulating  $\beta$ -catenin pathway activity. Thus, our data provide a new perspective on tumour-promoting roles of Circ\_0003945 in HCC, which might be a promising therapeutic target for HCC treatment.

Abnormal expression of circUBAP2 has been found in several cancer types, and a total of 90 circRNAs originating from UBAP2 have been identified, whereas specific back-splicing sites of these circRNAs still need to be further elucidated, and they might be derived from different exons. Meanwhile, few studies have explored the downstream signalling pathway induced by the circRNA/miRNA/mRNA axis. Here, we demonstrated that Circ\_0003945, a circUBAP2, plays the key role in HCC tumorigenesis and progression. Our study provides new evidence that Circ\_0003945 acts as a miR-34c-5p sponge and thus further protects  $\beta$ -catenin from phosphorylation, which may cause accumulation of  $\beta$ -catenin and trigger a series of gene transcription events. Taken together, our data comprehensively confirm that Circ\_0003945/miR-34c-5p/LGR4/ $\beta$ -catenin pathway exerts its tumour-promoting role in HCC progression. Interestingly,

Circ\_0003945 could be also sponged with miR-194-3p and upregulate MMP9 (one of the downstream target gene of  $\beta$ -catenin<sup>31</sup>), finally facilitating HCC progression.<sup>32</sup> Thus, Circ\_0003945/ $\beta$ -catenin axis might be a potential therapeutic target for HCC treatment.

Additionally, circRNAs are considered potential diagnostic and prognostic biomarkers.<sup>15,33-35</sup> A plasma circular RNA panel (hsa\_circ\_0000976, hsa\_circ\_0007750 and hsa\_circ\_0139897) was constructed to diagnose hepatitis B-virus-related HCC.<sup>36</sup> Although a previous study found that higher circUBAP2 expression can predict poor outcome for cervical cancer patients,<sup>37</sup> there is no data exploring the expression level of free circulating circUBAP2 in the circulation. In this study, elevated expression of Circ\_0003945 was identified in HCC tissue and correlated with HCC stage. Furthermore, a high level of plasma circulating Circ\_0003945 was confirmed in HCC patients compared with that in non-HCC groups. These findings imply that Circ\_0003945 might be a novel biomarker for HCC diagnosis and prognosis, as well as surveillance for treatment. However, a large-scale, prospective, multicentre study is still needed for further confirmation. It is noteworthy that we just focus on free circulating Circ\_0003945 level in this study, other sources for this circular RNA in the body should also be confirmed, such as whether it exists in exosomes. Accumulating evidence proposed that circRNAs could be enriched in exosomes and own potential diagnosis or prognostic value for HCC.<sup>35,38-40</sup> Therefore, the clinical performance of exosomal Circ\_0003945 needs to be elucidated in the future.

Although the tumour-promoting effects of Circ\_0003945 were verified to be as a miR-34c-5p sponge, other potential underlying mechanisms, such as interaction with proteins or translation into polypeptides, should also be considered. Considering that exosomal circular RNAs also play contribute to tumour progression,<sup>41-43</sup> the physiological function of exosomal Circ\_0003945 require further exploration.

In summary, we propose that Circ\_0003945 is significantly up-regulated in HCC cells and is related to HCC progression. Functionally and mechanistically, Circ\_0003945 might directly target miR-34c-5p to release its inhibition of LGR4 expression, finally activating the  $\beta$ -catenin pathway, and thus, enhancing malignant properties of HCC cell. Therefore, Circ\_0003945 might serve as a novel biomarker and therapeutic target for anti-HCC therapy.

#### ETHICS STATEMENT

The present study was approved by the Medical Ethics Committee of Zhongshan Hospital, Fudan University. All participants in this study provided written informed consent in accordance with the Declaration of Helsinki.

#### CONFLICT OF INTEREST

The authors declare no competing interests.

#### AUTHOR CONTRIBUTIONS

**Lihua Lyu:** Data curation (lead); Formal analysis (lead); Methodology (lead); Software (equal); Validation (lead); Visualization (lead); Writing – original draft (lead). **Chunyan Zhang:** Data curation (equal); Funding acquisition (equal); Supervision (equal). **Wenjing Yang:** Conceptualization (equal); Data curation (equal); Formal analysis (equal); Supervision (equal); Writing – original draft (equal). **Anli Jin:** Data curation (equal); Formal analysis (equal); Methodology (equal); Resources (equal); Software (equal). **Jie Zhu:** Data curation (equal); Formal analysis (equal); Resources (equal); Software (equal). **Hao Wang:** Data curation (equal); Formal analysis (equal); Software (equal); Writing – original draft (equal). **Te Liu:** Conceptualization (equal); Visualization (equal); Writing – original draft (equal); Writing – review & editing (equal). **Beili Wang:** Conceptualization (equal); Funding acquisition (equal); Project administration (equal); Visualization (equal); Writing – review & editing (equal). **Jianwen Cheng:** Data curation (equal); Methodology (equal); Resources (equal); Writing – original draft (equal). **Xinrong Yang:** Funding acquisition (equal); Supervision (equal); Validation (equal); Writing – review & editing (equal). **Wei Guo:** Conceptualization (lead); Funding acquisition (lead); Writing – review & editing (equal).

#### DATA AVAILABILITY STATEMENT

The datasets analysed for this study can be found in the Gene Expression Omnibus (GSE78520, GSE94508 and GSE97332).

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#### SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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