RESEARCH PAPER

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Salinomycin exerts anti-colorectal cancer activity by targeting the β-catenin/T-cell factor complex

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Background and Purpose: Salinomycin is a well-known inhibitor of human cancer stem cells (CSCs). However, the molecular mechanism(s) by which salinomycin targets colorectal CSCs is poorly understood. Here, we have investigated underlying antitumour mechanisms of salinomycin in colorectal cancer cells and three tumour models.

Experimental Approach: The inhibitory effect of salinomycin on the Wnt/β-catenin pathway was analysed with the SuperTopFlash reporter system. The mRNA expression of Wnt target genes was evaluated with real-time PCR. Effects of salinomycin on β-catenin/TCF4E interaction were examined using co-immunoprecipitation and an in vitro GST pull-down assay. Cell proliferation was determined by BrdU incorporation and soft agar colony formation assay. The stemness of the cells was assessed by sphere formation assay. Antitumour effects of salinomycin on colorectal cancers was evaluated with colorectal CSC xenografts, APC[min/+] transgenic mice, and patient-derived colorectal tumour xenografts.

Key Results: Salinomycin blocked β-catenin/TCF4E complex formation in colorectal cancer cells and in an in vitro GST pull-down assay, thus decreasing expression of Wnt target genes. Salinomycin also suppressed the transcriptional activity mediated by β-catenin/LEF1 or β-catenin/TCF4E complex and exhibited an inhibitory effect on the sphere formation, proliferation, and anchorage-independent growth of

colorectal cancer cells. In colorectal tumour xenografts and APC[min/+] transgenic mice, administration of salinomycin significantly reduced tumour growth and the expression of CSC-related Wnt target genes including LGR5.

Conclusions and Implications: Our study suggested that salinomycin could suppress the growth of colorectal cancer by disrupting the β-catenin/TCF complex and thus may be a promising agent for colorectal cancer treatment.

1 | INTRODUCTION

Colorectal cancer is the third most common malignancy and a major cause of cancer-related death worldwide. About 90% of colorectal cancers carry somatic mutations in Wnt signalling component genes such as the adenomatous polyposis coli (APC) and β-catenin (CTNNB1) genes, resulting in aberrant activation of the Wnt signalling pathway (Cancer Genome Atlas, 2012; Nusse & Clevers, 2017; Zhan, Rindtorff, & Boutros, 2017; Zhang & Shay, 2017). The protein β-catenin is a central component of the canonical Wnt signalling pathway. The stability of β-catenin is controlled by a cytoplasmic destruction complex that is composed of the APC tumour suppressor, the scaffolding protein Axin, glycogen synthase kinase 3β (GSK3β), and casein kinase 1 (CK1). APC binding to β-catenin leads to ubiquitin-mediated β-catenin degradation. Loss of APC function due to mutations stabilizes β-catenin, resulting in an accumulation of β-catenin in the cytosol as well as the nucleus, where it acts as a coactivator for the T-cell factor/lymphoid enhancing factor (TCF/LEF) transcription factors to activate the transcription of Wnt target genes (Clevers & Nusse, 2012). Indeed, nuclear β-catenin accumulation was detected in more than 80% of colorectal tumours and was significantly correlated with poor prognosis (Baldus et al., 2004; Sebio, Kahn, & Lenz, 2014; Wanitsuwan, Kanngurn, Boonpipattanapong, Sangthong, & Sangkhathat, 2008).

Wnt/β-catenin signalling is a crucial pathway of cancer stem cell (CSC) development. Its aberrant activation is essential for maintaining the self-renewal capacities of CSCs (de Sousa, Vermeulen, Richel, & Medema, 2011; Zeki, Graham, & Wright, 2011). There is good evidence for the presence of CSCs in colorectal cancer (Munro, Wickremesekera, Peng, Tan, & Itinteang, 2018) and CSCs are responsible for the tumour initiation, proliferation, chemoresistance, metastasis, and tumour recurrence. Targeting the CSC population may provide a new therapeutic strategy for colorectal cancer (Munro et al., 2018). Salinomycin, a monocarboxylic polyether antibiotic isolated from *Streptomyces albus*, is known to be an inhibitor of human CSCs. This compound selectively killed breast CSCs, at least 100 times more effectively than paclitaxel, in mice (Gupta et al., 2009). Salinomycin also selectively inhibited CSCs in a range of cancers, including breast, lung, gastric, osteosarcoma, squamous cell carcinoma, prostate, pancreatic, and colorectal cancers (Zhang, Li, Liu, Ma, & Chen, 2016). However, the molecular mechanisms by which salinomycin targets CSCs is poorly understood. Several potential mechanisms have been suggested (Dewangan, Srivastava, & Rath, 2017), including

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What is already known

- Salinomycin is a potent inhibitor of cancer stem cells.
- Salinomycin could inhibit Wnt/β-catenin signalling through targeting Wnt/LRP6 complex.

What this study adds

- Salinomycin could suppress the colorectal cancer growth by disrupting the β-catenin/TCF complex.

What is the clinical significance

- Salinomycin may be a promising therapeutic agent for colorectal cancers with APC or β-catenin mutation.

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suppression of the ATP-binding cassette (ABC) transporter (Fuchs, Daniel, Sadeghi, Opelz, & Naujokat, 2010; Kim et al., 2012) and blocking of pathways implicated in cancer such as Akt (Kuo et al., 2012), Wnt (D. Lu et al., 2011; D. Lu & Carson, 2011), Hedgehog (Y. Lu et al., 2015), and Notch (Zhou et al., 2014).

The anti-cancer activity of salinomycin against colorectal cancer has been demonstrated both in vitro and in vivo. Salinomycin selectively reduced the CD133^{+} cell subpopulations of colorectal cancer cells (Dong et al., 2011). Treatment with this compound decreased LRP6 protein levels and inhibited LRP6-phosphorylation in human CRC cells, resulting in the down-regulation of the Wnt target genes Fibronectin and leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5; Klose et al., 2016). These results suggest that the Wnt/β-catenin pathway was involved in the anti-cancer effect of salinomycin on colorectal cancers. As LRP6 is an upstream regulator of the Wnt pathway, it is questionable whether inhibiting LRP6 function is strong enough to interfere with Wnt/β-catenin signalling in human CRC cells exhibiting APC or β-catenin mutations. In the present study, we identified a novel mechanism for the inhibition of Wnt/β-catenin signalling by salinomycin in colorectal cancer. Salinomycin could inhibit Wnt/β-catenin signalling by disrupting the association between β-catenin and TCF4E. In colorectal CSC xenografts, APC[min/+] mice, and patient-derived colorectal tumour xenografts, salinomycin treatment significantly reduced tumour growth and the expression of CSC-related Wnt target genes including LGR5.

2 | METHODS

2.1 | Cell culture

HEK293T (Cat# CRL-3216; RRID:CVCL_0063), SW480 (Cat# CCL-228; RRID:CVCL_0546), HCT116 (Cat# CCL-247; RRID:CVCL_0291), and HT29 (Cat# HTB-38; RRID:CVCL_0320) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in DMEM (Thermo Fisher Scientific, Waltham, MA, USA; for HEK293T, HCT116, and HT29 cells) or IMDM (for SW480 cells; Thermo Fisher Scientific) supplemented with 10% FBS (Thermo Fisher Scientific) and 1% penicillin–streptomycin (Thermo Fisher Scientific) in a humidified incubator at 37°C with 5% CO_2.

2.2 | Luciferase reporter gene assays

Cells were transfected with SuperTopFlash or pDKK4-Luc reporter, the indicated expression plasmids and control plasmid pCMXβgal using the X-tremeGENE HP DNA Transfection Reagent (Roche Cat# 6366236001) according to the manufacturer's instructions. Cells were then treated with the indicated amounts of salinomycin. Cell lysates were used for the luciferase assay with a luciferase assay kit (Promega Cat# E1501, Madison, WI, USA), and the luciferase values were normalized according to β-galactosidase (β-gal) activity. Each treatment was performed in six replicates.

2.3 | Real-time PCR analyses

Total RNA was extracted using RNAiso Plus (TaKaRa Cat# 9109, Kusatsu, Shiga, Japan) and then reverse-transcribed into cDNA with the same RNA concentration for each sample using the Primescript RT Reagent Kit (TaKaRa Cat# RR037A, Kusatsu, Shiga, Japan) according to the manufacturer's instructions. Prepared cDNA was then subjected to quantitative PCR analysis using an ABI Prism 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with 2× SYBR Green qPCR Master Mix (Bimake Cat# B21203, Houston, TX, USA). Real-time PCR assays were performed to quantify mRNA levels of human Axin2, CD44, cyclin D1, DKK1, c-Myc, LEF1, LGR5, and Sox2 genes. The comparative Ct method was used to analyse relative expression of genes. The data are presented as the fold change. The fold change was calculated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{treated} - \Delta Ct_{control}$. Ct is the cycle number at which fluorescence first exceeds the threshold. The ΔCt values from each target gene were obtained by subtracting the values for GAPDH Ct from the sample Ct. The data from five independent experiments and three technical replicates per sample are presented. The primer sequences are shown in Table S1.

2.4 | Immunoblot analyses

The antibody-based procedures used in this study comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018). Cells or tumour tissues were lysed in lysis buffer containing 0.1-M Tris–HCl (pH 7.0), 2% SDS, 10% glycerol, 0.1-mM DTT, 1-mM EDTA, 1-mM EGTA, 2.5-mM sodium pyrophosphate, 1-mM β-glycerol phosphate, 1-mM sodium orthovanadate, 2 µg·ml^{-1} leupeptin, and 1-mM PMSF, followed by sonication. Proteins were fractionated by SDS-PAGE and transferred to PVDF membranes (Cat# ISEQ00005, Millipore, Burlington, MA, USA). Western blotting was performed with the following primary antibodies: anti-β-catenin (1:2,000, Santa Cruz Biotechnology Cat# sc-7963, RRID:AB_626807), anti-TCF4E (1:2,000, Cell Signaling Technology Cat# 2569, RRID:AB_2199816), anti-LGR5 (1:1,000, Abcam Cat# ab75732, RRID:AB_1310281), anti-CD44 (1:3,000, Cell Signaling Technology Cat# 3570, RRID:AB_2076465), anti-Sox2 (1:1,000, Cell Signaling Technology Cat# 23064, RRID:AB_2714146), anti-Flag (1:5,000, Sigma-Aldrich Cat# F1804, RRID:AB_262044), anti-V5 (1:5,000, Cell Signaling Technology Cat# 13202, RRID: AB_2687461), anti-GST (1:5,000, Cell Signaling Technology Cat# 2625, RRID:AB_490796), anti-His (1:5,000, Proteintech Group Cat# 10001–0-AP, RRID:AB_11232228), anti-GAPDH (1:5,000, Transgen Biotech Cat# HC301–02, RRID:AB_2629434), and anti-β-actin (1:5,000, Transgen Biotech Cat# HC-201-02) at 4°C overnight. Then the PVDF membranes were incubated with HRP conjugated goat anti-mouse (1:10,000, Thermo Fisher Scientific Cat# A16066, RRID: AB_2534739) or anti-rabbit (1:10,000, Thermo Fisher Scientific Cat# A16096, RRID:AB_2534770) IgG for 1 hr at room temperature. After incubated with ECL Plus Western Blotting Substrate (Thermo Fisher Scientific Cat# 32132), the immunoblots were developed by either X-ray film (Kodak, Rochester, NY) or Chemiluminescent Imaging System (Tanon 5200, Shanghai, China). All immunoblot assays were performed using five independent samples. Densitometric analysis was carried out using the Quantity One 1-D Analysis Software, and the quantification results were normalized to the loading control.

2.5 | Co-immunoprecipitation

Total protein lysate of cells or tumour tissues was extracted with lysis buffer (20-mM Tris–HCl, pH 7.4, 150-mM NaCl, 1-mM EDTA, 1-mM EGTA, 1% Triton X-100, 2.5-mM sodium pyrophosphate, 1-mM β-glycerol phosphate, 1-mM sodium orthovanadate, 2 µg·ml^{-1} leupeptin, and 1-mM PMSF). The supernatant fractions were separated by centrifugation at 13,523× g for 15 min and subject to immunoprecipitation using the anti-Flag M2 affinity gel (Sigma-Aldrich Cat# A2220, RRID:AB_10063035) or anti-β-catenin conjugated sepharose beads (Cell Signaling Technology Cat# 12475, RRID: AB_2797931). The data from five independent experiments are presented.

2.6 | Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed using the ChIP-IT Express Enzymatic Chromatin Immunoprecipitation

Kit (Active Motif Cat# 53035) according to the manufacturer's instructions. The eluted DNA was amplified by PCR with the primers as previous indicated (Fang et al., 2016). This was followed by analysis with real-time PCR. The results from five independent experiments are presented. The antibodies used were anti-β-catenin (Santa Cruz Biotechnology Cat# sc-7963, RRID: AB_626807) and anti-mouse IgG (Cell Signaling Technology Cat# 5415, RRID:AB_10829607).

2.7 | Immunofluorescence staining

Immunofluorescence staining was performed as previously described (Wang et al., 2014). Briefly, cells were fixed with 4% paraformaldehyde and permeabilized with 0.4% Triton X-100. Following blocking, cells were incubated with anti-β-catenin antibody (1:200, Santa Cruz Biotechnology Cat# sc-7963, RRID: AB_626807) for 2 hr at room temperature. Alexa Fluor 488 conjugated goat anti-mouse IgG antibodies (1:200, Thermo Fisher Scientific Cat# A-11001, RRID:AB_2534069) were used as secondary antibody. To visualize the cell nucleus, DAPI was used. Sections were observed with a Leica laser scanning confocal microscope (Leica TCS SP5II, Wetzlar, Germany). The acquisition settings were as follows: DAPI, excitation/emission (nm): 358/461, β-catenin, excitation/emission (nm): 495/519. The images were analysed by Leica LAS AF lite software (Leica Application Suite X, RRID:SCR_013673).

2.8 | BrdU cell proliferation assays

Cells were plated on 96-well plates and then treated with the indicated concentrations of salinomycin for 24 hr. The BrdU incorporation assay was performed using the Cell Proliferation ELISA BrdU Colorimetric Kit (Roche Cat# 11669915001) according to the manufacturer's instructions. Each treatment was performed in six replicates.

2.9 | Anchorage-independent growth assays

A six-well plate was pre-coated with 1.5-ml 0.7% agar in a complete medium per well. The cells were harvested, suspended in 0.35% agar in a complete medium with the indicated amounts of salinomycin and then plated in triplicate onto the pretreated six-well plate (1.5-ml medium with 3,000 cells per well). The cells were then cultured in a humidified incubator at 37°C with 5% CO_2 for 2 weeks. Colonies were photographed following staining with crystal violet, and only those colonies larger than 0.05-mm were counted. Each treatment was performed in six replicates.

2.10 | Sphere formation assays

SW480 or HT29 cells were seeded at 250 cells per well in the DMEM/F12 medium (2% B-27, 10 ng·ml^{-1} EGF, 10 ng·ml^{-1} FGF, and 10 μg·ml^{-1} insulin) with the indicated amounts of salinomycin in a 24-well plate with an Ultra-Low Attachment surface. After 10 days

of incubation, spheres with diameter over 50 μm were counted, and representative fields were microphotographed. Each treatment was performed in six replicates.

2.11 | Human colorectal cancer tissue samples

The colorectal cancer tissues were collected after all patients signed the written informed consent, in accordance with the Human Research Ethics Committee of Shenzhen University, permit number 201619008 (Approved on November 15, 2016). Five patients who had endoscopic biopsies with histologically confirmed colorectal cancer in the First Affiliated Hospital of Shenzhen University were included in this study. None of the patients had been previously treated with radiotherapy and chemotherapy.

2.12 | Animal model studies

Animal care and experiments were performed in accordance with the Animal Research Ethics Committee of Shenzhen University, permit number AEWC-201412003 (Approved on December 25, 2014). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010; McGrath & Lilley, 2015) and with the recommendations made by the *British Journal of Pharmacology*.

NCG mice (Stock# T001475) were purchased from GemPharmatech Co. Ltd., Nanjing, China. B6-APC$^{min/+}$ mice (Stock# T001457) were purchased from Nanjing Biomedical Research Institute of Nanjing University, Nanjing, China. NPG mice were purchased from Vitalstar Biotechnology, Beijing, China. All mice were housed in a specific pathogen free facility with six mice per cage under a 12-hr light/12-hr dark cycle at a constant temperature of 24°C and fed a standard rodent diet in the laboratory animal research centre of Shenzhen University. The animals were acclimatized to the laboratory for at least 1 week prior to the start of the experiments. All surgery procedures were performed under anaesthesia using isoflurane. At the end of the experiments, animals were humanely killed by CO_2 asphyxiation. In animal studies, both the carer of the animals and the assessor of the results are blinded.

To generate colorectal CSC xenografts, SW480 cells were stained with FITC-conjugated anti-human CD44 antibody (eBioscience Cat# 11-0441-81, RRID:AB_465044) and APC-conjugated anti-human CD133 antibody (BD Biosciences Cat# 566596, RRID:AB_2744280), and CD44^{high}CD133^{high} cells were collected as colorectal CSCs using BD FACSAria™ III Fluorescence Activated Cell Sorter (BD FACSARIA III cell sorter, RRID:SCR_016695) as previously described (Wang et al., 2012). Then 5,000 CD44^{high}CD133^{high} SW480 cells were subcutaneously injected into 8-week-old male NCG mice (n = 12). Three days later, mice were randomly divided into two groups (n = 6 per group) and treated with the vehicle (0.8% DMSO/12% Cremophor/8% ethanol in normal saline; n = 6) or 5 mg·kg^{-1} salinomycin in vehicle (n = 6) by intraperitoneal injection every 3 days. Tumour size and body weight were measured every 3 days. After

treatment for 1 month, mice were killed, and the tumours were dissected, weighed, and photographed.

Twelve 8-week-old male B6-APC[min/+] mice were randomly divided into two groups ($n = 6$) and treated with the vehicle (0.8% DMSO/12% Cremophor/8% ethanol in normal saline) or 5 mg·kg^{−1} salinomycin in vehicle twice weekly by intraperitoneal injection. Animals were weighed and checked weekly. At the age of 14 weeks, the mice were killed. The colon was collected and photographed before being fixed in buffered formalin.

Establishment of patient-derived colorectal tumour xenografts (PDTXs) was performed as previous described (Gao et al., 2015). Briefly, five patient-derived colorectal tumour pieces were implanted subcutaneously into the five 8-week-old male NPG mice. After successful expansion of the F1–F3 generations, ~1 mm[3] of PDTX tumour fragments (F3) were transplanted into the right flank of 8-week-old male NPG mice. To evaluate the effect of salinomycin on PDTX growth, tumour fragments from each PDTX were implanted into two male NPG mice. Tumour growth was closely observed and measured every 3 days. When the tumours reached ~50 mm[3], the two mice implanted with tumour fragments from five patients were randomly divided into two groups and intraperitoneally injected with the vehicle (0.8% DMSO/12% Cremophor/8% ethanol in normal saline) or 5 mg·kg^{−1} salinomycin in vehicle every 3 days. Subsequently, tumour volumes were measured with a calliper and calculated using the following formula: $0.52 \times length \times width^2$. After treatment for 36 days, the mice were killed, and the tumour tissues were collected and weighed before being fixed in buffered formalin.

2.13 | Histological analyses

As described previously, formalin-fixed tumours were embedded with paraffin and sectioned; after which, immunohistochemistry and H&E staining were performed. Immunohistochemistry was performed using the following primary antibodies: anti-human Ki-67 (1:200, BioLegend Cat# 350501, RRID:AB_10662749), anti-mouse Ki-67 (1:200, Cell Signaling Technology Cat# 12202, RRID: AB_2620142), anti-LGR5 (1:100, Abcam Cat# ab75732, RRID: AB_1310281), anti-human CD44 (1:100, Cell Signaling Technology Cat# 3570, RRID: AB_2076465), and anti-mouse CD44 (1:100, Abcam Cat# ab157107).

2.14 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). All experiments were randomized and blinded. The normal probability plot was used to examine data distributions. Student's *t* test was applied when the data showed normal distribution. Statistical analyses were carried out with GraphPad prism7.00 software (GraphPad, RRID: SCR_000306). The data were analysed by Student's *t* test or one-way ANOVA followed by Dunnett's *t* test. Results are presented as mean ± *SD*. Differences at $P < .05$ were considered statistically significant.

2.15 | Materials

Salinomycin was obtained from Sigma-Aldrich (Cat # 563080-M). The SuperTopFlash reporter vector was kindly provided by Karl Willert (University of California at San Diego, San Diego, CA, USA). The pDKK4-Luc reporter was constructed by cloning a DKK4 promoter region (−542 to −1) into the luciferase reporter vector pGL3-basic (Su et al., 2018). The expression plasmids encoding β-catenin, LEF1, TCF4E, and β-galactosidase (β-gal) have been described previously (Wang et al., 2016). The expression plasmid encoding N-terminal mutant of β-catenin (β-catenin 4A) was generated by site-directed mutagenesis (Easy Mutagenesis System, Transgen Biotech Cat# FM111-02) according to the manufacturer's instructions, The resulting plasmid was designated as pcDNA3/β-catenin 4A in which N-terminal residues Ser-33, Ser-37, Thr-41, and Ser-45 have been mutated to Ala. GST-tagged β-catenin, GST-tagged β-catenin (134–668), and His-tagged TCF4E (1–79) were constructed as previously reported (Fang et al., 2016).

2.16 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMA-COLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Christopoulos et al., 2017; Alexander, Fabbro et al., 2017; Alexander, Kelly et al., 2017).

3 | RESULTS

3.1 | Salinomycin inhibits β-catenin-mediated signalling in colorectal cancer cells

To examine the effect of salinomycin on Wnt/β-catenin signalling in colorectal cancer cells, colorectal cancer cells (SW480 and HCT116) were transfected with SuperTopFlash reporter. Treatment with 0.25- to 5-μM salinomycin dose-dependently inhibited the transcriptional activity of the SuperTopFlash reporter, with IC$_{50}$ values at 3.12 μM in SW480 cells and 0.22 μM in HCT116 cells (Figure 1a,b). Furthermore, salinomycin down-regulated transcription of the Wnt target genes Axin2, CD44, cyclin D1, DKK1, c-Myc, and LEF1 in both cell lines (Figure 1c,d). It has been well documented that APC is deleted at the carboxyl terminus at residue 1338 in SW480 cells, and HCT116 cells have a S45 mutation in β-catenin. These results suggest that salinomycin may act downstream of APC or β-catenin in colorectal cancer cells. To validate this hypothesis, HEK293T cells were
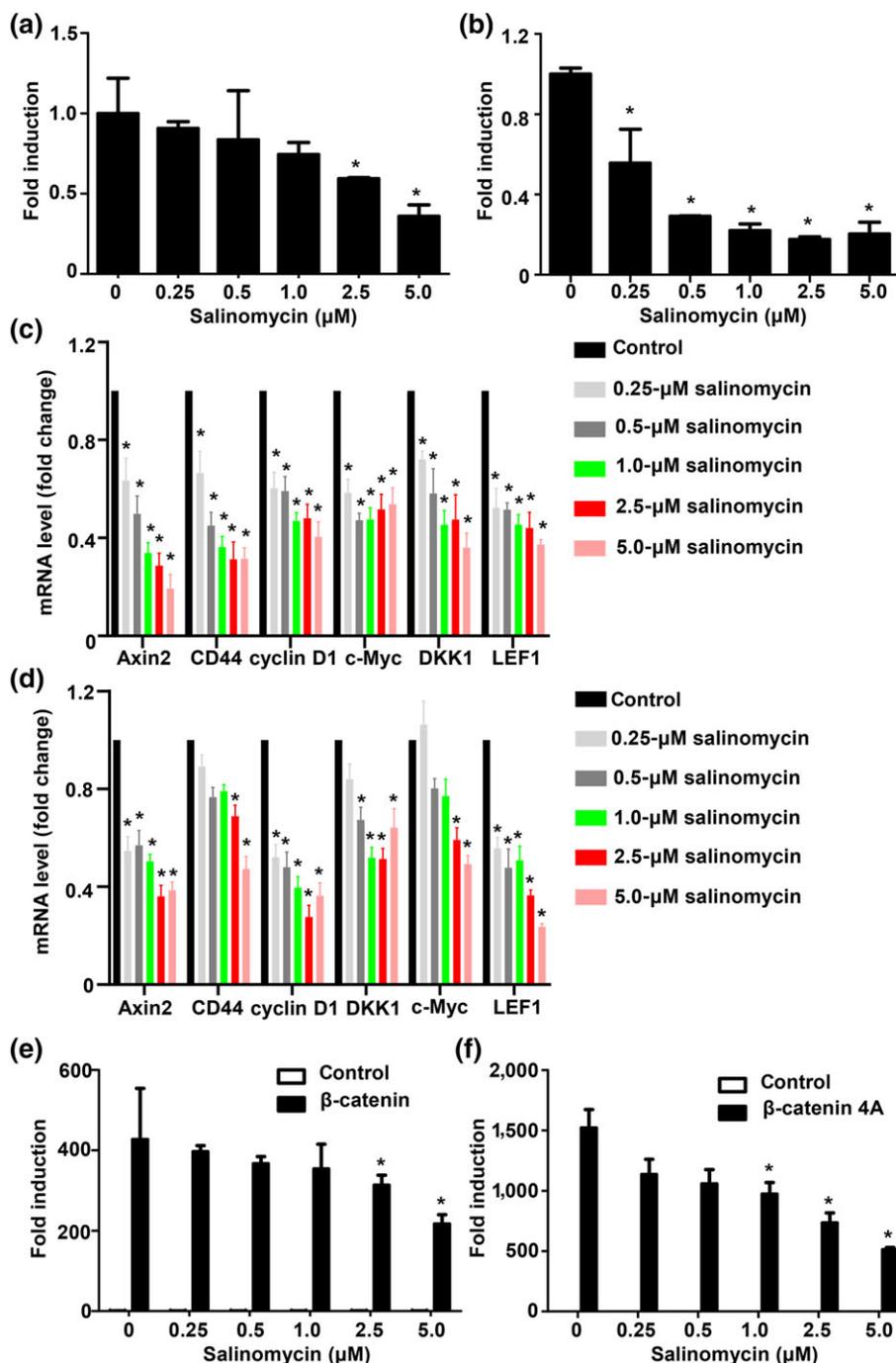
FIGURE 1 Salinomycin directly represses Wnt/β-catenin signalling. (a, b) The SuperTopFlash reporter was transfected into colorectal cancer cells SW480 (a) or HCT116 (b). The cells were then incubated with DMSO or salinomycin (0.25–5.0 μM) for 24 hr. The luciferase values were normalized to β-gal activities. Each treatment was performed in six replicates ($n = 6$). (c, d) SW480 (c) or HCT116 (d) cells were treated with the indicated amounts of salinomycin for 24 hr, and then total RNA was extracted and reverse-transcribed into cDNA. Real-time PCR analysis was used to detect the mRNA expression of Wnt target genes Axin2, CD44, cyclin D1, DKK1, c-Myc, and LEF1. The data from five independent experiments are presented ($n = 5$). (e, f) HEK293T cells were transfected with the SuperTopFlash reporter along with an empty vector or expression plasmids encoding wild type β-catenin (e) or β-catenin 4A (f). The transfected cells were then treated with the indicated concentrations of salinomycin for 24 hr. The luciferase values were normalized to β-gal activities. Each treatment was performed in six replicates. Values shown are means ± SD ($n = 6$). *$P < .05$, significantly different from the vehicle control; one-way ANOVA followed by Dunnett's t test.

transfected with a SuperTopFlash reporter plasmid together with wild type or N-terminal mutant of β-catenin (β-catenin 4A). As expected, salinomycin treatment effectively suppressed Wnt signalling induced by wild type β-catenin (Figure 1e) or β-catenin 4A (Figure 1f), with IC$_{50}$ values at 6.07 μM for wild type β-catenin and 2.0 μM for β-catenin 4A.

3.2 | Salinomycin has little effect on the localization and the expression level of β-catenin in colorectal cancer cells

We further evaluated the effect of salinomycin on the localization and the expression level of β-catenin in colorectal cancer cells. SW480 and HCT116 cells were treated with increasing concentrations of salinomycin (0.25–5 μM) for 24 hr. As shown in Figure 2a,b, salinomycin had little effect on the expression level of β-catenin in both cell lines. Additionally, immunofluorescence staining showed that salinomycin did not affect the localization of β-catenin in colorectal cancer cells (Figure 2c,d).

3.3 | Salinomycin disrupts the association between β-catenin and TCF4E

To assess whether salinomycin affects β-catenin binding to the Wnt target gene promoter, the ChIP assay was used to detect β-catenin binding to the promoters of two known Wnt target genes, Axin2 and c-Myc, in SW480 cells. As shown in Figure 3a, salinomycin significantly reduced β-catenin binding to Axin2 and c-Myc promoters (Figure 3a).

As DNA-bound TCF/LEF transcription factors are required to recruit β-catenin to a promoter, we investigated the effect of salinomycin on the interaction between β-catenin and TCF4E or LEF1 using immunoprecipitation. Expression plasmids for Flag-β-catenin 4A and TCF4E-V5 (Figure 3b) or LEF1-V5 (Figure 3c) were transfected into HEK293T cells, and cell extracts were collected for affinity purification by anti-Flag M2 agarose. As shown in Figure 3b, c, Flag-β-catenin 4A was specifically coprecipitated with TCF4E-V5 or LEF1-V5, and the interactions were dose-dependently inhibited after salinomycin treatment (Figure 3b,c).

We then examined the effect of salinomycin on the interaction of endogenous β-catenin and TCF4E proteins in colorectal cancer cells. SW480 (Figure 3d), HCT116 (Figure 3e), and HT29 (Figure 3f) cells were incubated with salinomycin (0.25–5 μM) for 24 hr and then lysed and subject to co-immunoprecipitation with anti-β-catenin antibody. The results showed that the anti-β-catenin antibody specifically precipitated endogenous β-catenin and TCF4E. Treatment with salinomycin reduced the β-catenin/TCF4E interaction in a dose-dependent manner in all three colorectal cancer cell lines (Figure 3d–f).

To further confirm the inhibitory effect of salinomycin on the interaction between β-catenin and TCF4E, a GST pull-down assay was performed. The expression plasmids for GST-tagged full-length β-catenin
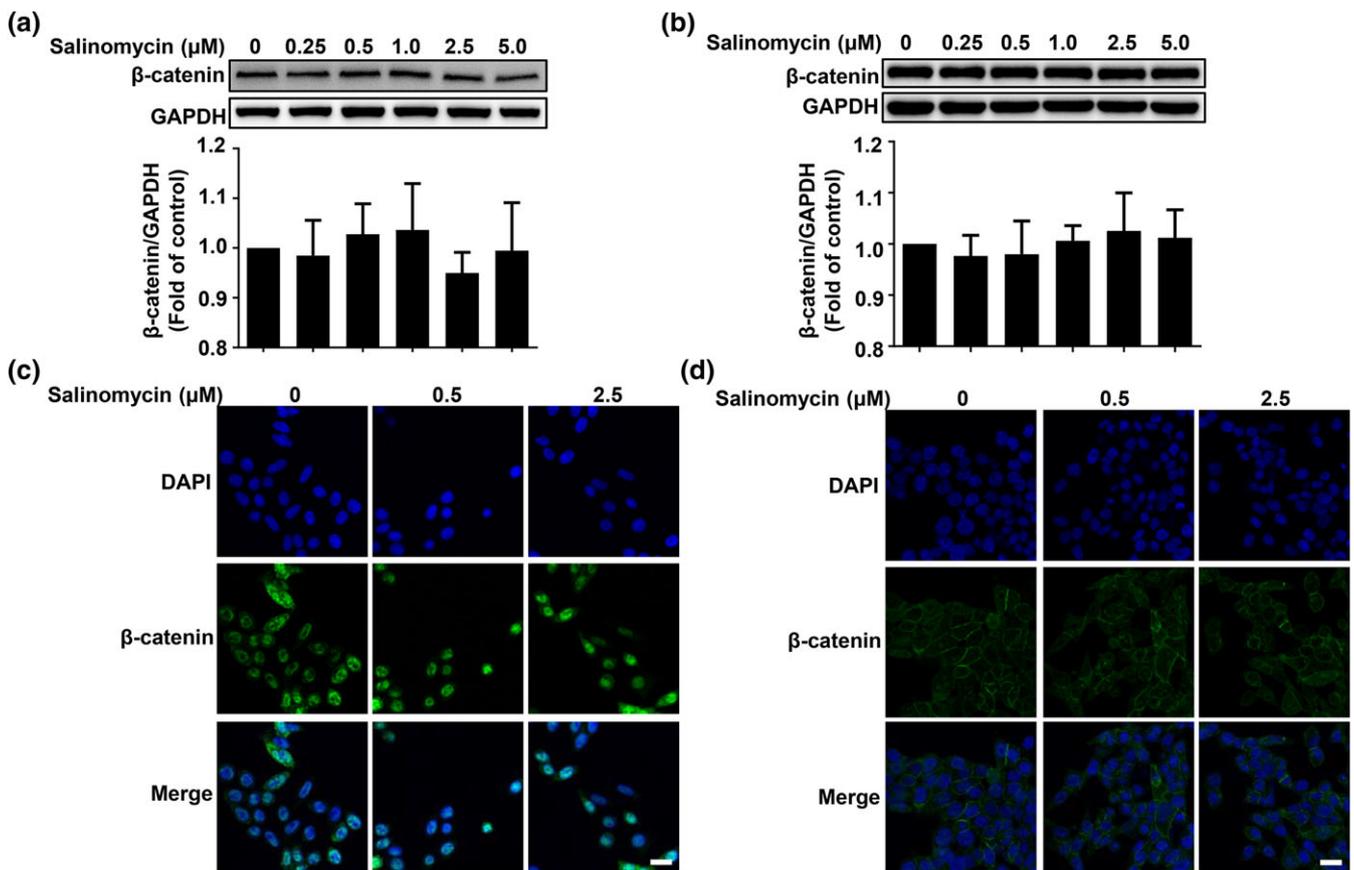

FIGURE 2 The expression and translocation of β-catenin are not affected by salinomycin. (a, b) The colorectal cancer SW480 (a) or HCT116 (b) cells were treated with vehicle or salinomycin (0.25–5.0 μM) for 24 hr. The expression level of β-catenin was detected by immunoblots. The lower panel represents the densitometric quantification of the protein bands (n = 5). Values are means ± SD. (c, d) SW480 (c) or HCT116 (d) cells were treated with the indicated concentrations of salinomycin for 24 hr; after which, the cells were fixed and followed by immunofluorescent staining. Scale bar, 20 μm (c and d)
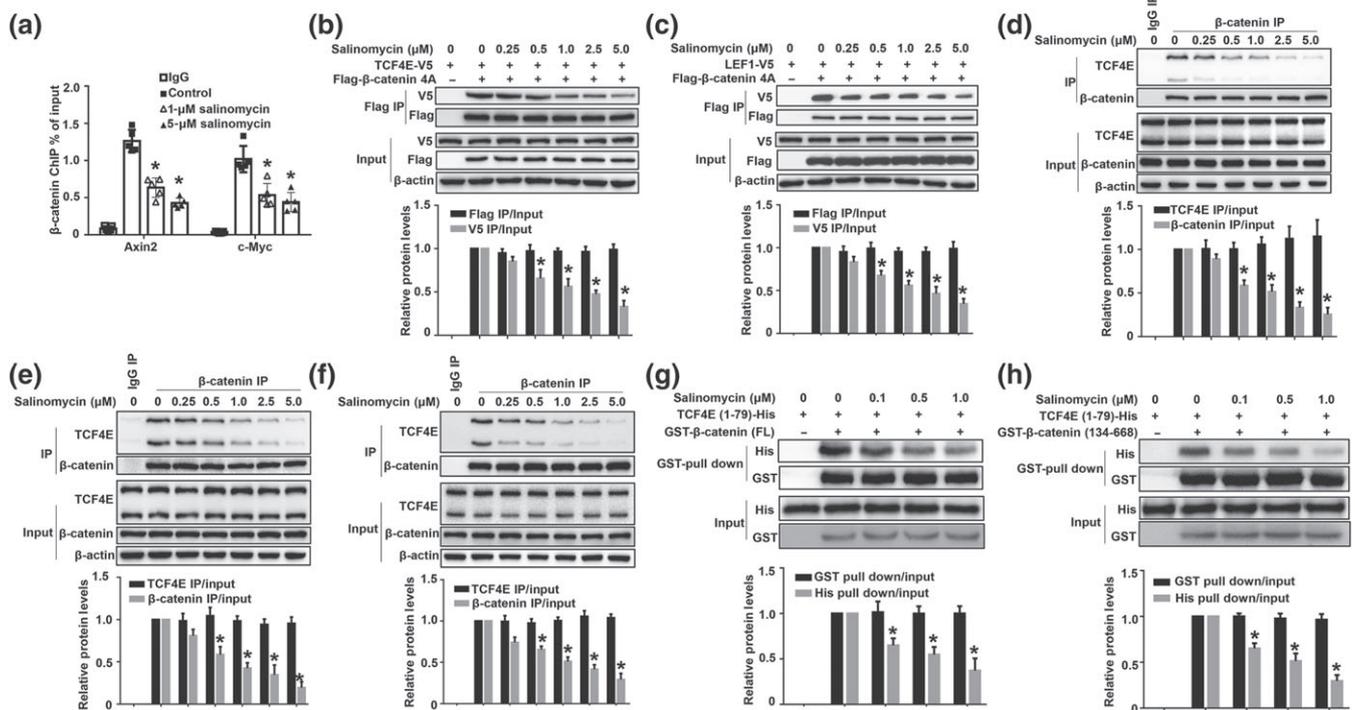
FIGURE 3 Salinomycin interferes with the interaction of β-catenin and TCF4E. (a) SW480 cells were treated with 2.5-μM salinomycin for 24 hr, and a ChIP assay was performed using the control IgG or anti-β-catenin antibody. Real-time PCR analysis was used to detect the binding of β-catenin to the Axin2 and c-Myc promoters. The data from five independent experiments are presented (n = 5). (b, c) The expression plasmids for Flag-β-catenin 4A and TCF4E-v5 (b) or LEF1-v5 (c) were transfected into HEK293T cells. The cells were then treated with the indicated amounts of salinomycin for 24 hr before immunoprecipitation was carried out by anti-Flag M2 beads. The interaction between β-catenin 4A and TCF4E (B) or LEF1 (C) was visualized by immunoblots. The lower panel represents the densitometric quantification of the protein bands (n = 5). (d–f) The colorectal cancer SW480 (d), HCT116 (e), or HT29 (f) cells were incubated with DMSO or salinomycin (0.25–5.0 μM) for 24 hr, and then immunoprecipitation was performed using control IgG or β-catenin antibody. The interaction between β-catenin and TCF4E was visualized using immunoblots. The lower panel represents the densitometric quantification of the protein bands (n = 5). (g) Purified GST-tagged β-catenin was incubated with His-tagged TCF4E (1–79) in the presence or absence of the indicated amounts of salinomycin. The interaction between β-catenin and TCF4E (1–79) was visualized using immunoblots. The lower panel represents the densitometric quantification of the protein bands (n = 5). (h) Purified GST-tagged armadillo domain of β-catenin (134–668) was incubated with His-tagged TCF4E (1–79) in the presence or absence of the indicated amounts of salinomycin. The interaction between armadillo domain of β-catenin (134–668) and TCF4E (1–79) was visualized using immunoblots. The lower panel represents the densitometric quantification of the protein bands (n = 5). Values shown are means ± SD (n = 5). *P < .05, significantly different from the vehicle control; one-way ANOVA followed by Dunnett's t test (a–h).

(GST-β-catenin), GST-tagged armadillo repeat domain of β-catenin (GST-β-catenin 134–668), and His-tagged N-terminal region of TCF4E (His-TCF4E 1–79) were constructed and expressed in *Escherichia coli*, respectively. GST-β-catenin and GST-β-catenin (134–668) were purified with GST-Sepharose and incubated with purified His-TCF4E (1–79). The results showed that the full-length β-catenin (Figure 3g) or armadillo repeat domain of β-catenin (Figure 3h) interacted with the N-terminal region of TCF4E and addition of salinomycin dose-dependently inhibited the interaction between two proteins.

3.4 | Salinomycin suppresses the transcriptional activity mediated by β-catenin/LEF1 or β-catenin/TCF4E

To test the effect of salinomycin on the transcriptional activity induced by β-catenin/LEF1 or β-catenin/TCF4E, the pDKK4-Luc reporter was constructed by cloning the DKK4 promoter region, which contains five putative TCF-binding sites, into a luciferase reporter vector. DKK4 is a target gene of Wnt/β-catenin signalling. The activation of a pDKK4-Luc reporter gene requires the simultaneous presence of β-catenin and LEF1 or TCF4E (Bazzi, Fantauzzo, Richardson, Jahoda, & Christiano, 2007; Su et al., 2018). HEK293T cells were transfected with the pDKK4-Luc reporter together with expression vectors encoding β-catenin 4A, LEF1, TCF4E, β-catenin 4A/LEF1, and β-catenin 4A/TCF4E, respectively. As expected, β-catenin 4A/LEF1 or β-catenin 4A/TCF4E strongly activated the transcriptional activity of pDKK4-Luc reporter, while β-catenin 4A or LEF1 or TCF4E alone had a much weaker effect on the reporter gene transcription (data not shown). Treatment with salinomycin suppressed the reporter activity induced by either β-catenin 4A/LEF1 (Figure 4a) or β-catenin 4A/TCF4E (Figure 4b) in a dose-dependent fashion, with IC50 values at 4.4 μM for β-catenin 4A/LEF1 and 3.86 μM for β-catenin 4A/TCF4E.
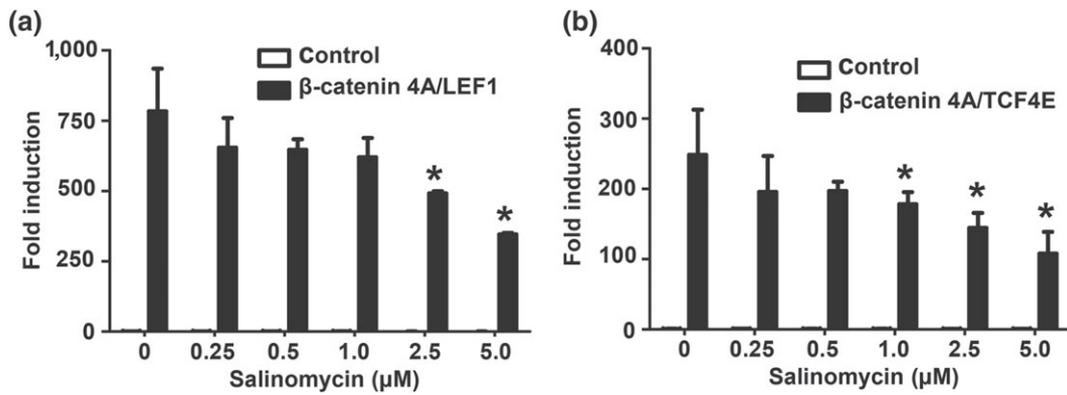
FIGURE 4 The transcriptional activity mediated by β-catenin/LEF1 or β-catenin/TCF4E is repressed by salinomycin. (a) HEK293T cells were transfected with the pDKK4-Luc reporter along with an empty vector or expression plasmids for β-catenin 4A/LEF1 as indicated. The cells were then incubated with DMSO or salinomycin (0.25–5.0 μM) for 24 hr. The luciferase values were normalized to β-gal activities. Each treatment was performed in six replicates ($n = 6$). (b) HEK293T cells were transfected with the pDKK4-Luc reporter along with an empty vector or expression plasmids for β-catenin 4A/TCF4E as indicated. The cells were then treated with the indicated amounts of salinomycin for 24 hr. The luciferase values were normalized to β-gal activities. Each treatment was performed in six replicates. Values shown are means ± SD ($n = 6$). *$P < .05$, significantly different from the vehicle control; one-way ANOVA followed by Dunnett's t test (a, b).
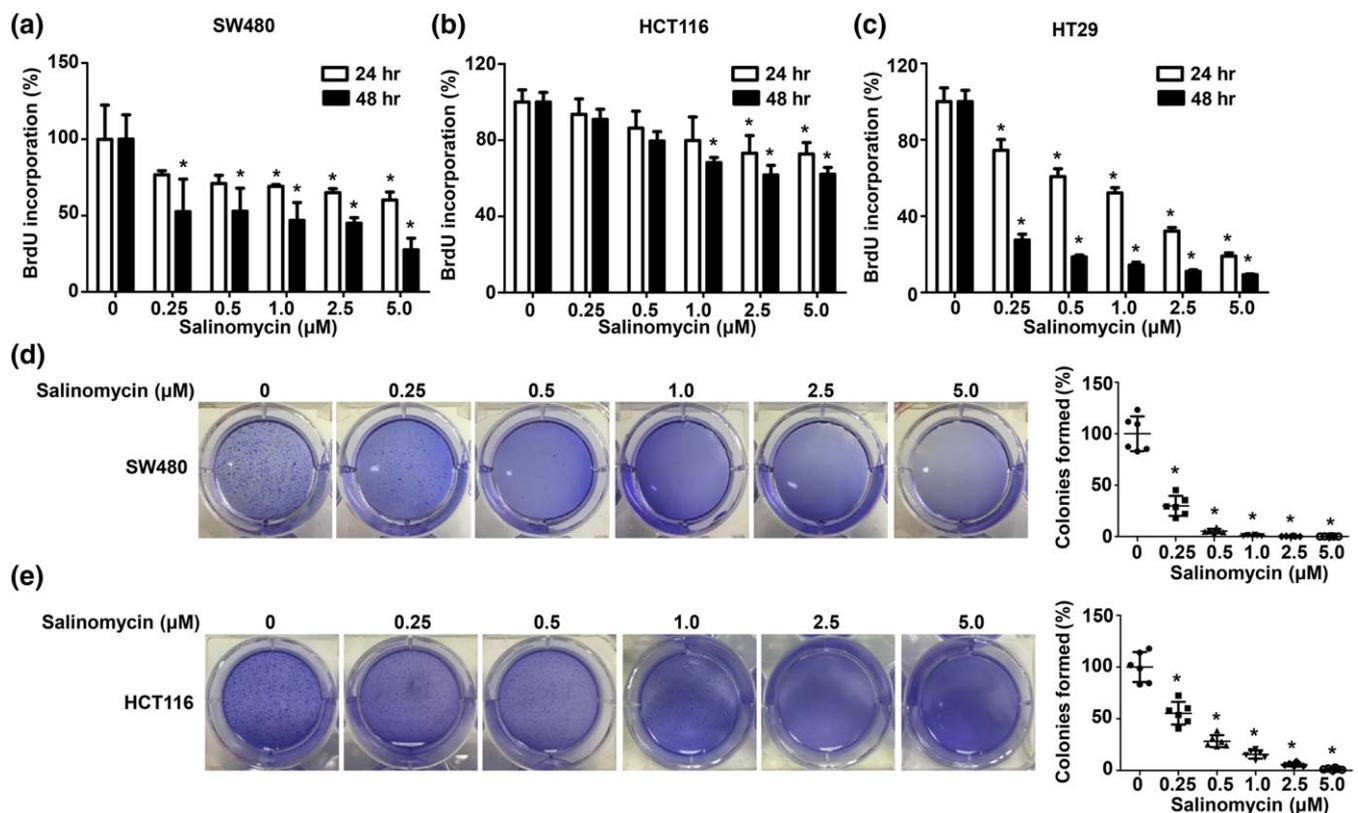

FIGURE 5 Salinomycin significantly suppresses proliferation and anchorage-independent growth in colorectal cancer cells. (a–c) SW480 (a), HCT116 (b), and HT29 (c) cells were treated with the indicated amounts of salinomycin for 24 hr, and then BrdU incorporation assay was used to examine cell proliferation. Each treatment was performed in six replicates ($n = 6$). (d, e) The anchorage-independent growth of SW480 (d) and HCT116 (e) cells was detected using a soft agar colony formation assay. Right panel: Graphical representation of quantitative data shows the relative number of colonies formed, as shown in (d) and (e). Each treatment was performed in six replicates. Individual values are shown with means ± SD ($n = 6$). *$P < .05$, significantly different from the vehicle control; one-way ANOVA followed by Dunnett's t test (a–e).

3.5 | Salinomycin inhibits the proliferation and anchorage-independent growth of colorectal cancer cells

To examine the effect of salinomycin on the proliferation of colorectal cancer cells, a BrdU incorporation assay was performed. Treatment with salinomycin resulted in a dose-dependent reduction in BrdU incorporation in SW480 (Figure 5a), HCT116 (Figure 5b), and HT29 (Figure 5c) cells, with IC_{50} values at 24 hr of 29.56 μM and 48 hr of 0.57 μM in SW480 cells, at 24 hr of 33.67 μM and 48 hr of 9.23 μM in HCT116 cells, and at 24 hr of 0.96 μM and 48 hr of 0.03 μM in HT29 cells. A soft agar colony formation assay was then used to investigate the effect of salinomycin on the anchorage-independent growth of colorectal cancer cells. Salinomycin treatment significantly decreased the colony number of SW480 cells (Figure 5 d) and HCT116 cells (Figure 5e). These results indicate that salinomycin exerts an inhibitory effect on the proliferation and anchorage-independent growth of colorectal cancer cells.

3.6 | Salinomycin inhibits colorectal cancer cell stemness

Salinomycin is the first agent being identified as a selective inhibitor of CSCs (Gupta et al., 2009). To explore the effect of salinomycin on the stemness of colorectal cancer cells, a sphere formation assay was performed. The colorectal cancer SW480 and HT29 cells were incubated with salinomycin at 0.5 and 1.0 μM for 1 week, and sphere formation was markedly inhibited after treatment with salinomycin (Figure 6a,b). The expression of stemness marker genes, LGR5, CD44, and Sox2, was then examined by real-time PCR. The results showed that salinomycin decreased mRNA expression of these stemness marker genes (Figure 6c,d). As expected, salinomycin significantly suppressed the protein levels of LGR5, CD44, and Sox2 in SW480 and HT29 cells (Figure 6e,f). Having established that the expression of LGR5, CD44, and Sox2 genes could be up-regulated by activation of Wnt/β-catenin signalling (Barker et al., 2007; Van Raay et al., 2005; Wielenga et al., 1999), salinomycin-induced
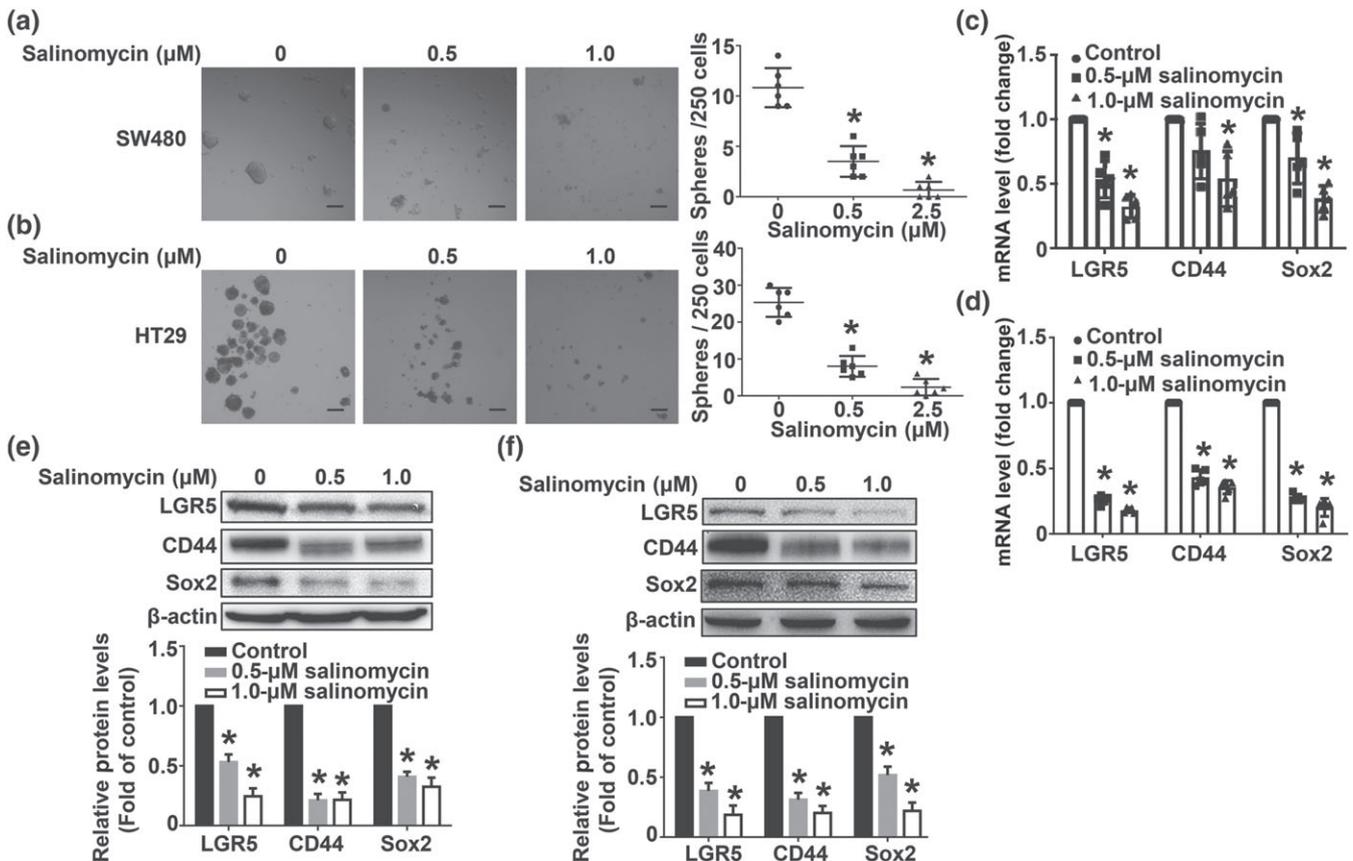

FIGURE 6　Salinomycin inhibits the sphere-forming ability and the expression of stemness marker genes in colorectal cancer cells. (a, b) SW480 (a) and HT29 (b) cells were cultured in Ultra-Low Attachment dishes to examine the ability of sphere formation in the absence or presence of salinomycin at 0.5 and 1.0 μM. Scale bar, 100 μm (a and b). Right panel: Graphical representation of quantitative data shows the relative number of spheres formed as shown in (a) and (b). Each treatment concentration was performed in six replicates (n = 6). (c, d) SW480 (c) and HT29 (d) cells were incubated with the indicated amounts of salinomycin for 24 hr and mRNA levels of stemness marker genes LGR5, CD44, and Sox2 were measured using real-time PCR. The data from five independent experiments are presented (n = 5). (e, f) SW480 (e) and HT29 (f) cells were incubated with the indicated amounts of salinomycin for 24 hr, and immunoblots were performed to detect the protein levels of stemness marker genes LGR5, CD44, and Sox2. The lower panel represents the densitometric quantification of the protein bands (n = 5). Values shown are means ± SD. *P < .05, significantly different from the vehicle control; one-way ANOVA followed by Dunnett's t test (a–f).

inhibition of stemness is most likely associated with its antagonistic effects on Wnt/β-catenin signalling.

3.7 | Salinomycin represses the growth of colorectal CSC xenografts

To evaluate the effect of salinomycin on the in vivo tumour-seeding ability of colorectal CSCs, CD44^{high}/CD133^{high} SW480 cells were implanted subcutaneously in NCG mice. Three days later, mice were randomly divided into two groups and treated with vehicle control and salinomycin for 1 month. Our results showed that tumour volumes in the salinomycin-treated group decreased significantly compared to the control group (Figure 7), indicating that salinomycin could suppress the in vivo tumour-seeding ability of colorectal CSCs.

3.8 | Salinomycin attenuates colorectal tumour growth by blocking Wnt/β-catenin signalling in APC[min/+] transgenic mice

To evaluate the in vivo efficacy of salinomycin against colorectal cancer, APC[min/+] transgenic mice were employed. These animals bear a mutation at codon 850 of the APC gene. Over time, these mice spontaneously generate numerous adenomatous polyps in the small intestine and fewer polyps in the colon within several weeks of birth. Eight-week-old mice were randomly divided into two groups and treated with either vehicle control or 5 mg·kg^{-1} salinomycin twice a week for 6 weeks. The mice were then killed, and the entire gastrointestinal tract was dissected out. Salinomycin treatment did not affect

body weight of mice. Figure 8a,b showed that salinomycin significantly reduced the size and number of colonic polyps (Figure 8a,b). Histological analyses revealed that salinomycin treatment reduced tumour cell density (Figure 8c) and proliferation as indicated by Ki-67 staining (Figure 8d). Immunohistochemical staining was used to detect the expression of Wnt signalling target molecules LGR5 and CD44. Salinomycin-treated tumours showed significantly decreased levels of LGR5 and CD44 compared with vehicle-treated tumours (Figure 8e,f). These results suggest that salinomycin suppresses colonic polyp growth by targeting Wnt signalling in APC[min/+] mice.

3.9 | Salinomycin suppresses colorectal tumour growth and antagonizes β-catenin/TCF-mediated signalling in patient-derived colorectal tumour xenografts

The antitumour activity of salinomycin was assessed using five unique patient-derived colorectal tumour xenograft (PDTX) models. For each PDTX model, the patient-derived colorectal tumour pieces were implanted subcutaneously into the NPG mice. Expansion of the F1–F3 generations was carried out. The tumour pieces from PDTX (F3) were minced into small pieces and implanted into the NPG mice. Tumour fragments from each PDTX were implanted into two mice. After transplantation, mice were randomized into vehicle or salinomycin groups when tumour volumes reached about 50 mm^3. Mice were treated with vehicle or salinomycin at 5 mg·kg^{-1} every 3 days. Tumour size and body weight were monitored every 3 days. Mice were killed 36 days after the treatment, and the tumours were
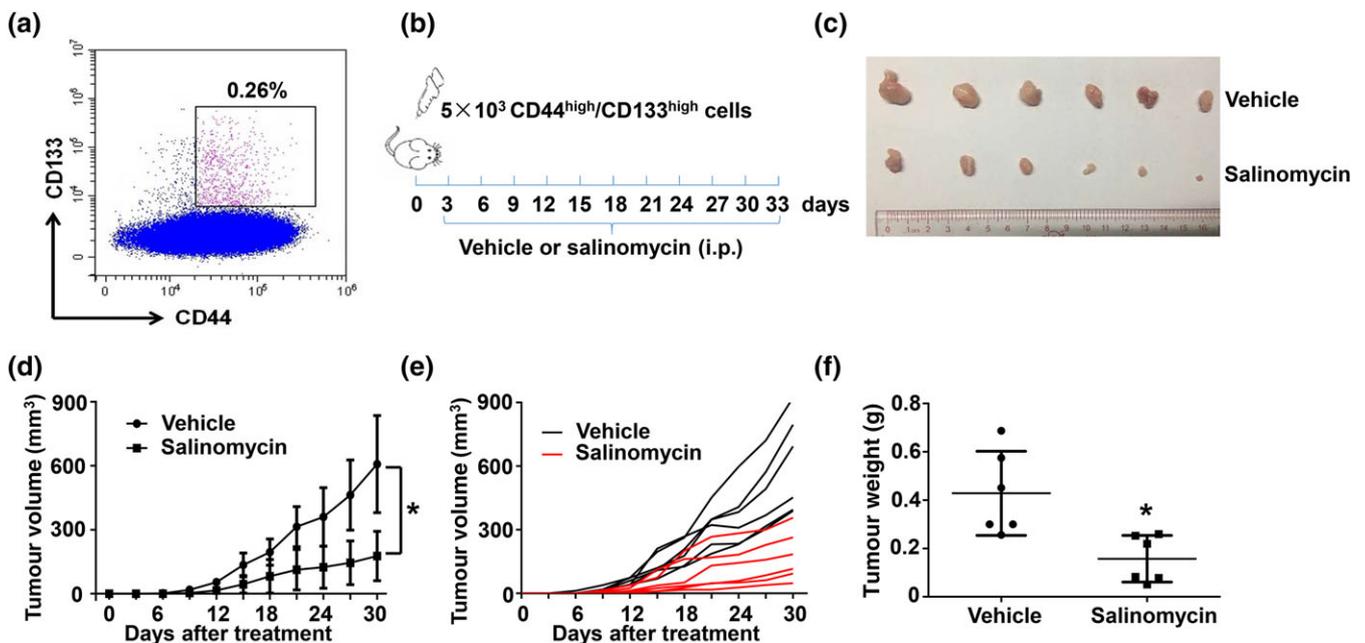

FIGURE 7 Salinomycin inhibits the growth of tumours in colorectal CSC xenograft mice. (a) CD44^{high}/CD133^{high} SW480 cells were isolated as CSCs by FACS. (b) The scheme of SW480 CSC inoculation and salinomycin treatment. (c) Images of tumours from the control group and salinomycin-treated group. (d) Mean tumour volume (n = 6). (e) Tumour volume. Each coloured line represents an individual mouse. (f) Mean tumour weight (n = 6). Values are means ± SD. *P < .05, significantly different from the vehicle control; one-way ANOVA followed by Dunnett's t test (d) and Student's t test (f).
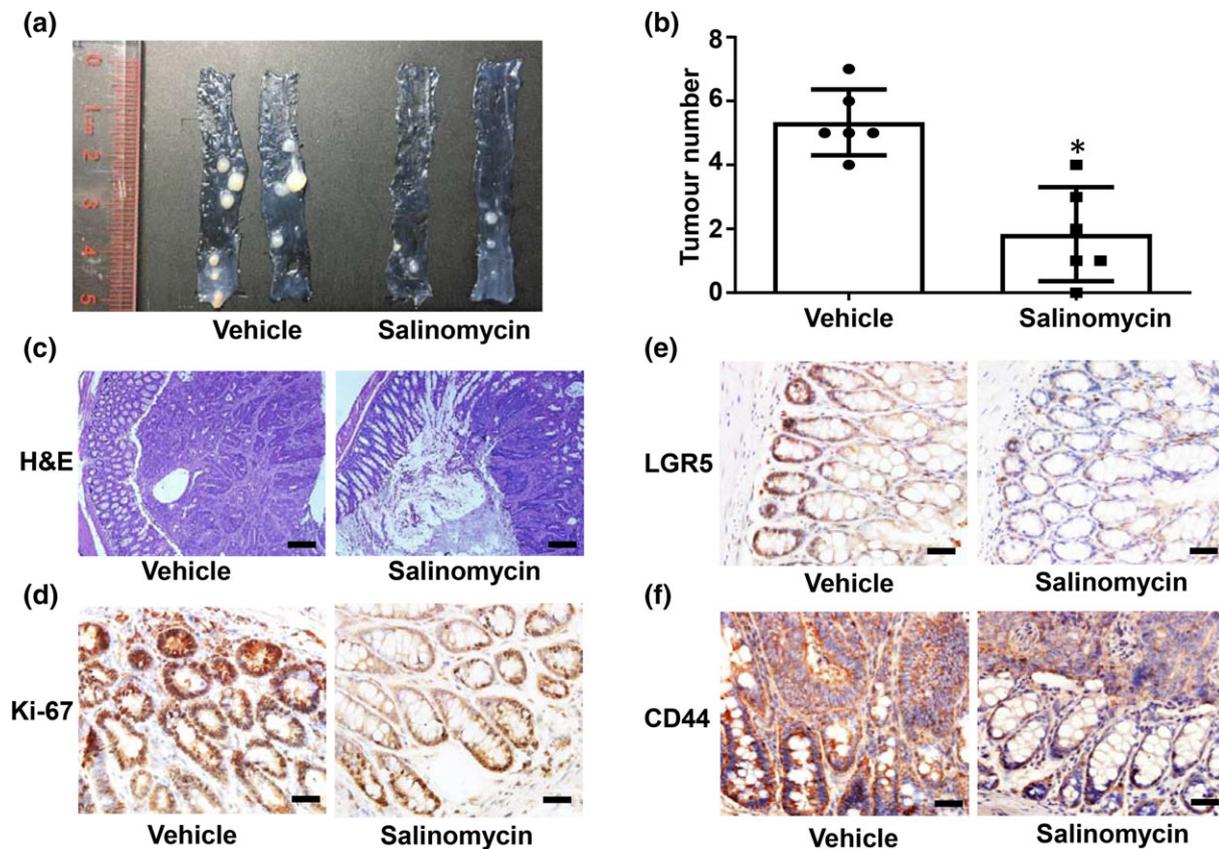
FIGURE 8 Inhibitory effect of salinomycin on the development of colonic tumours in APC$^{min/+}$ mice. (a) Representative image of colonic tumours from the control group and salinomycin-treated group. (b) Graphical representation of quantitative data shows the number of colonic tumours from the control group and salinomycin-treated group. Individual values are shown with means ± SD (n = 6). *P < .05, significantly different from the vehicle control; Student's t test. (c) H&E staining; scale bar, 200 μm. (d) Ki-67 antibody staining. (e) LGR5 antibody staining. (f) CD44 antibody staining; scale bar, 50 μm (d–f).

dissected. The results showed that salinomycin effectively inhibited the growth of colorectal tumours compared with the vehicle-treated group (Figure 9a–c). Treatment with salinomycin also decreased tumour cell density and expression of the proliferation marker Ki-67 (Figure 9d,e). There was no significant change in mouse body weight due to salinomycin treatment, suggesting minimal toxicity.

To examine the effect of salinomycin on the binding of β-catenin to TCF4E in colorectal cancer tissues, a specific β-catenin antibody was employed for the co-immunoprecipitation in tumour tissue lysates from control and salinomycin-treated groups. Compared with those in control group, the co-immunoprecipitated TCF4E protein levels were significantly reduced in the tumour tissues of salinomycin-treated group, suggesting that salinomycin could inhibit the association of β-catenin with TCF4E in colorectal tumour tissues (Figure 9f). Moreover, real-time PCR results showed that salinomycin treatment down-regulated the transcription of Wnt target genes Axin2, cyclin D1, DKK1, c-Myc, and LEF1 (Figure 9g).

We further examined the effect of salinomycin on the expression of stemness marker genes LGR5, CD44, and Sox2 in patient-derived colorectal tumour xenografts. These genes are known to be regulated by Wnt/β-catenin signalling. Real-time PCR analysis showed a decrease in mRNA expression of these genes in salinomycin-treated

tumours (Figure 9g). Consistent with these findings, salinomycin treatment noticeably decreased the protein levels of LGR5 (Figure 9h,j), CD44 (Figure 9i,j), and Sox2 (Figure 9j).

4 | DISCUSSION

Aberrant activation of the Wnt/β-catenin pathway is the most important driving force in human colorectal cancer. Mutations in components of the Wnt signalling pathway, including APC and β-catenin, lead to β-catenin stabilization and the formation of nuclear β-catenin/TCF4 complex (Nusse & Clevers, 2017). Constitutive activation of Wnt signalling may favour the growth and maintenance of colorectal CSCs (Batlle & Clevers, 2017). The β-catenin/TCF4 complex could also impose a crypt progenitor phenotype on colorectal cancer cells (van de Wetering et al., 2002). Vermeulen et al. (2010) showed that colorectal CSCs exhibit high activity of the Wnt/β-catenin signalling pathway. Kanwar, Yu, Nautiyal, Patel, and Majumdar (2010) reported that colonospheres formed by colon cancer cell lines are highly enriched in CSCs and that the Wnt/β-catenin pathway plays a critical role in growth and maintenance of colonospheres. Thus, targeting Wnt/β-catenin signalling in colorectal CSCs holds great
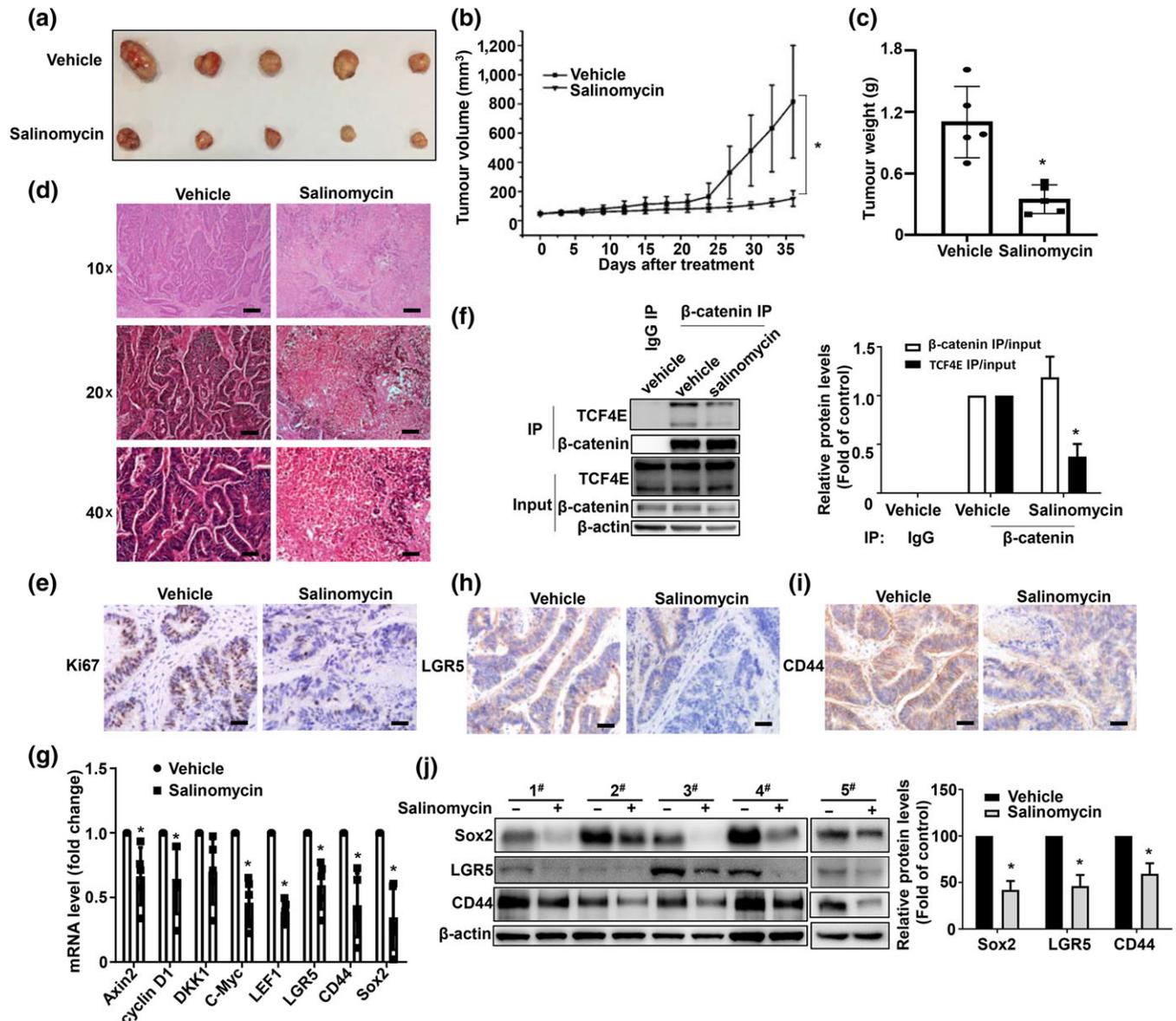
FIGURE 9 Salinomycin inhibits tumour growth in the patient-derived xenograft mouse model. (a) Images of tumours from the control group and salinomycin-treated group. (b) Mean tumour volume ($n = 5$). (c) Mean tumour weight ($n = 5$). (d) H&E staining. Scale bar, 200 μm (10×), scale bar, 100 μm (20×), scale bar, 50 μm (40×). (e) Ki-67 staining; scale bar, 50 μm. (f) Proteins were extracted from tumour samples, and immunoprecipitation was performed using control IgG or anti-β-catenin antibody. The interaction between β-catenin and TCF4E was visualized by immunoblots. The graphical panel represents the densitometric quantification of the protein bands ($n = 5$). (g) Total RNA was extracted from tumour samples, and real-time PCR was used to determine the mRNA levels of Wnt target genes and stemness-associated genes ($n = 5$). (h) LGR5 antibody staining; scale bar, 50 μm. (i) CD44 antibody staining; scale bar, 50 μm. (j) The expression levels of Sox2, LGR5, and CD44 in tumour samples were visualized after immunoblotting. Graphical representation of quantitative data shows the relative expression of Sox2, LGR5, and CD44 ($n = 5$). Values are means ± SD. *$P < .05$, significantly different from the vehicle control; one-way ANOVA followed by Dunnett's t test (b) and Student's t test (c, f, g, and j)

promise for successful therapy. However, no FDA-approved drugs are currently available that directly target the Wnt/β-catenin signalling pathway of CSCs for the treatment of colorectal cancer (Kahn, 2014; Lyou, Habowski, Chen, & Waterman, 2017).

In this study, we have demonstrated that salinomycin suppressed colorectal cancer cell stemness by interfering β-catenin/TCF4E transcriptional complexes. Salinomycin also inhibited the growth of xenograft tumours derived by colorectal CSC population in mice.

Furthermore, salinomycin reduced Wnt-driven intestinal tumourigenesis in APC$^{min/+}$ mice. Additionally, administration of salinomycin significantly decreased the growth of the patient-derived colorectal tumour xenografts established from the five patients, accompanied by down-regulation of CSC-related Wnt target genes LGR5, CD44, and Sox2. These results highlighted the anti-cancer effect of salinomycin on colorectal cancer and present its potential in the treatment of colorectal cancers with mutations in the APC or CTNNB1 gene.

Our group and other groups have demonstrated that salinomycin could inhibit proximal Wnt signalling by blocking LRP6 phosphorylation and inducing its degradation (Dewangan et al., 2017; D. Lu et al., 2011; W. Lu & Li, 2014). In our previous studies, micromolar concentrations of salinomycin were needed to block Wnt signalling induced by downstream activators, such as β-catenin, suggesting additional mechanisms must be involved in the inhibitory effect of salinomycin on Wnt signalling (D. Lu et al., 2011). In colorectal cancers, mutations in APC and CTNNB1 genes are two major factors for aberrant activation of Wnt/β-catenin signalling. We thus explored the effect of salinomycin on the β-catenin/TCF4E transcriptional complex in colorectal cancer cells. Our results showed that salinomycin could disrupt association between β-catenin and TCF4E. Moreover, treatment with salinomycin suppressed the transcriptional activity of pDKK4-Luc reporter activated by either β-catenin 4A/LEF1 or β-catenin 4A/TCF4E in a dose-dependent fashion. Taken together, our results revealed a novel mechanism underlying the inhibitory effect of salinomycin on Wnt/β-catenin signalling in colorectal cancers. This study provides a rational basis for the therapeutic potential of salinomycin in cancers with mutational activation of the β-catenin destruction complex, such as colorectal carcinomas.

Although the colorectal cancer cell lines, SW480 and HCT116, contain mutated β-catenin, β-catenin exhibits a distinct subcellular localization in both cell lines. In HCT116 cells, β-catenin staining was strongest at the membrane with diffuse staining throughout the cytoplasm and nucleus, whereas β-catenin localized predominantly to the nucleus with weak cytoplasmic staining in SW480 cells, which is consistent with previous studies (Han et al., 2013), suggesting that β-catenin subcellular distribution is cell line dependent. As β-catenin is known to interact with a number of proteins, β-catenin localization may be associated with expression and location of its interacting proteins. However, treatment with salinomycin had no effect on β-catenin subcellular localization in either cell line.

LGR5 is a target of Wnt/β-catenin signalling and considered a functional CSC marker in human colorectal cancers, which makes it an attractive target for the development of CSC-directed therapeutics (Hirsch & Ried, 2016; Shimokawa et al., 2017). LGR5^{+} colorectal cancer cells can serve as CSCs in growing cancer tissues. The expression of LGR5 is positively associated with poor prognosis in colorectal cancers (Chen et al., 2014). LGR5^{high} colorectal cancer cells exhibit higher clonogenic potential in vitro as well as higher tumourigenicity in vivo when compared to the LGR5^{low} cancer cells (Kemper et al., 2012). In addition to LGR5, several other Wnt target genes have been proposed as colorectal CSC markers including CD44 and Sox2 (Voutsadakis, 2015). In this study, we observed decreased expression of stemness marker genes LGR5, CD44, and Sox2 in colorectal cancer cells, in response to salinomycin treatment. Salinomycin also suppressed sphere formation of colorectal cancer cells. Importantly, salinomycin reduced tumour growth in colorectal CSC xenografts, APC[min/+] mice, and patient-derived colorectal tumour xenografts and the expression of stemness marker genes LGR5 and CD44 at both mRNA and protein levels in APC[min/+] mice and patient-derived colorectal tumour xenografts. These results indicated that salinomycin

could selectively suppress colorectal CSCs through targeting Wnt/β-catenin signalling.

In a pilot study, salinomycin has been used for the treatment of patients with metastasized breast, ovarian, and head and neck cancer. Salinomycin was intravenously administered every second day for 3 weeks, resulting in inhibition of disease progress for months without any serious long-term side effects (Naujokat & Steinhart, 2012). Salinomycin at 4 mg·kg^{-1}·day^{-1} showed effective antitumour activity in a patient-derived mouse xenograft model of colorectal cancer (Klose et al., 2019). Moreover, intraperitoneal injection of salinomycin at doses of 3 and 5 mg·kg^{-1}·day^{-1} markedly suppressed tumour growth in human gastric cancer xenografts without causing obvious toxicities (Li et al., 2016). However, adverse effects of salinomycin have also been observed. Ojo, Bhadauria, and Rath (2013) reported that intraperitoneal administration of salinomycin at 1, 3, or 5 mg·kg^{-1} for 28 days had dose-dependent adverse effects on male reproductive organs and fertility in mice. Mice treated with 5 mg·kg^{-1} were generally well tolerated. Pharmacokinetic evaluation revealed that the serum concentrations of salinomycin could reach to 1.716 ± 0.233 μM in mice treated with 5 mg·kg^{-1} salinomycin (Boehmerle, Muenzfeld, Springer, Huehnchen, & Endres, 2014). Our study showed that 0.25- to 5-μM salinomycin dose-dependently inhibited the transcriptional activity of the SuperTopFlash reporter, suggesting that salinomycin-induced antagonism of the Wnt/β-catenin signalling pathway occurs at comparable concentrations producing therapeutic and toxic effects.

The major obstacle for clinical application of salinomycin is its systemic toxicity in vivo, with a narrow margin of safety (Dewangan et al., 2017). Our results revealed great potential of salinomycin for treatment of colorectal cancer. Recently, site-specific colon drug delivery has been developed for improving treatment of colon-related diseases, while minimizing systemic toxicity (Amidon, Brown, & Dave, 2015). The colon has several advantages for drug delivery, such as near neutral pH, long transit time, relatively low activity of proteolytic enzymes and increased responsiveness to absorption enhancers. Approaches used for colon-specific delivery include pro-drugs, pH sensitive polymers, timed release delivery systems, and microbially degraded delivery systems (Philip & Philip, 2010). We believe that colon-targeted drug delivery will considerably facilitate the clinical application of salinomycin to human colorectal cancer. Further studies are needed to develop colon-targeted salinomycin delivery systems for treatment of colorectal cancer.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

Z.W., L.Z., D.A.C., and D.L. developed the concept and methodology. Z.W., L.Z., Y.X., S.Y., H.L., J.F., F.L., Z.S., J.S., Q.S., S.L., Y.X., L.Z., S.L., and D.L. performed the experiments, carried out the data acquisition, analysed the study. Z.W., L.Z., P.H., F.G., D.A.C., and D.L. did the manuscript preparation.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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