β -catenin regulates IRF3-mediated innate immune signalling in colorectal cancer

Chengming Ding^{1,2,3} | Jun He² | Jun Zhao³ | Junhua Li³ | Jie Chen¹ | Wenyan Liao⁴ | Yi Zeng⁵ | Jing Zhong⁶ | Chaoying Wei⁷ | Liming Zhang⁷ | Mei Zhou⁷ | Zeming Jia¹ | Yaoting Zhang¹ | Hui Li⁷ | Yuzheng Zhou⁷ | Xiaolong Xiao⁷ | Dong Han² | Chong Li² | Zhu Zhu² | Zanzian Xia^{7,8,9} | Jian Peng¹

¹Department of General Surgery, Xiangya Hospital, Central South University, Changsha, China

²Department of Hepatopancreatobiliary Surgery, The First Affiliated Hospital, University of South China, Hengyang, China

³Department of Molecular Microbiology and Immunology, Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, CA, USA

⁴Department of Obstetrics and Gynecology, The First Affiliated Hospital, University of South China, Hengyang, China

⁵Department of Pathology and Immunology, Youjiang Medical University for Nationalities, Baise, China

⁶Institute of Clinical Medicine, The First Affiliated Hospital, University of South China, Hengyang, China

⁷The State Key Laboratory of Medical Genetics and School of Life Sciences, Central South University, Changsha, China

⁸Hunan Fangsheng Pharmaceutical Co. Ltd., Changsha, China

⁹Sinobioway 3C Translational Medicine Co. Ltd., Changsha, China

Correspondence

Jian Peng, Department of General Surgery, Xiangya Hospital, Central South University, Changsha, China.

Email: pengjian1698@aliyun.com

Zanzian Xia, The State Key Laboratory of Medical Genetics and School of Life Sciences, Central South University, Changsha, China.

Email: xiazanzian@sklmg.edu.cn

Funding information

the Joint Funds of the National Natural Science Foundation of China, Grant/Award Number: U1603126; Project of China Scholarship Council, Grant/Award Number: 201406370025; the National Key Research and Development Program of China, Grant/Award Number: 2016YFC1200200 and 2016YFD0500300; Hunan Provincial Natural Science Fund, Grant/Award Number: (2018JJ3471) and 2017JJ2334;

Abstract

Objective: β -catenin is one of the most critical oncogenes associated with many kinds of human cancers, especially in the human CRC. Innate immunity recognizes tumour derived damage-associated molecular patterns (DAMPs) and primes the anti-tumour adaptive responses. While the function of β -catenin in CRC tumourigenesis is well established, its impact on innate immune evasion is largely unknown. The aim of this study is to characterize the role of β -catenin in inhibiting RIG-I-like receptor (RLR)-mediated IFN- β signalling in colorectal cancer.

Materials and Methods: Immunohistochemical staining and western blotting were conducted to study the expression of β -catenin, IRF3 and phospho-IRF3 (p-IRF3) in CRC samples and cell lines. Plaque assay determining virus replication was performed to assess the regulation of β -catenin on IFN- β signalling. The inhibition of β -catenin on RLR-mediated IFN- β signalling was further studied by real-time analyses and reporter assays in the context of lentiviral-mediated β -catenin stably knocking down. Lastly, co-immunoprecipitation and nuclear fractionation assay were conducted to monitor the interaction between β -catenin and IRF3.

Results: We found that high expression of β -catenin positively correlated with the expression of IRF3 in CRC cells. Overexpression of β -catenin increased the viral replication. Conversely knocking down of β -catenin inhibited viral replication. Furthermore, our data demonstrated that β -catenin could inhibit the expression of

IFN- β and interferon-stimulated gene 56 (ISG56). Mechanistically, we found that β -catenin interacted with IRF3 and blocked its nuclear translocation.

Conclusion: Our study reveals an unprecedented role of β -catenin in enabling innate immune evasion in CRC.

1 | INTRODUCTION

The highly conserved β -catenin protein, the vertebrate's homologue of *Drosophila armadillo*, is one of the most frequent proto-oncogenes. β -catenin is composed of 781 amino acids which forms 12 armadillo repeats. It is widely expressed in normal tissues, such as cardiac muscle, and aberrantly overexpressed in various human cancers including colorectal, hepatocellular, lung, breast, ovarian and endometrial cancers.¹ Among these cancers, the tumorigenic role of β -catenin-mediated activation of the canonical WNT signalling pathway which passes signals into a cell through cell surface receptors and then leads to regulation of gene transcription, is well recognized in human CRC.^{2,3} In addition, β -catenin also participates in the regulation of other signalling pathways, such as NF- κ B signalling and TGF- β signalling.^{4,5} Recently, emerging evidences suggested that β -catenin was also involved in the regulation of innate and adaptive immunity in mouse liver injury.⁶ All of these observations prompted us to hypothesize that β -catenin might regulate immune responses during CRC development.

Immune surveillance plays crucial roles in controlling tumorigenesis and cancer progression.^{7,8} An important aspect of malignant tumour progression is the immune evasion. Tumour cells are able to escape from the detection and destruction by the adaptive arms of the immune system through numerous strategies, including the downregulation of MHC molecules, the secretion of immune suppressive molecules like PD-L1 and so on.^{9,10} On the other hand, innate immune evasion by tumour cells remains poorly characterized. From the host perspective, innate immune responses represent the first line of the defence which detect microbial pathogens or other dangerous signals via a set of pattern-recognition receptors (PRRs) that can recognize damage or pathogen-associated molecular patterns (PAMPs).¹¹ Tumour associating nucleotides or metabolites will be sensed by PRRs including the RIG-I-like receptor (RLR) family which can recognize double-stranded RNA or single-stranded RNA,¹² the membrane-associated Toll-like receptors (TLRs)¹³ which can recognize different double-stranded RNA or single-stranded RNA internalized into the endosomes of specified cells,⁷ and more importantly, DNA sensors including cGAS and DAI, which serve as cytosolic sensors for microbial DNA or leaking DNA from tumours.¹⁴⁻¹⁶ Once activated upon recognition, these pattern-recognition receptors will trigger the downstream signalling cascades that result in a rapid production of type I interferons and pro-inflammatory cytokines.¹⁷ In the context of cancers, these cytokines will prime and cooperate with adaptive immune responses which together eradicate the abnormal cancer cells. Ample evidences have proved that the type I interferon, interferons- β (IFN- β) directly inhibits tumorigenesis by

regulating tumour cell proliferation and apoptosis in human melanoma, hepatocellular carcinoma and breast cancers.¹⁸⁻²⁰

The central transcription factor, interferon-regulatory factor 3 (IRF3), is mainly responsible for the induction of interferon- β (IFN- β) and essential for the activation of interferon responses.²¹ Upon detecting the pathogen by PRR, IRF3 is phosphorylated by TBK1 kinase complex and undergoes a rapid conformational change, allowing its homo-dimerization and nuclear translocation where it binds to the interferon-stimulated response elements of the target genes.²² The important role of IRF3 in the induction of interferon responses suggests that it must be appropriately regulated and "finely tuned" to determine the accurate nature of the final innate immune responses.²³ On the other hand, cancer cells might hijack these regulatory mechanisms or even develop novel strategies to block the interferon responses, in fulfilling the escape of the immune surveillance.

Here, we report a novel function of β -catenin in regulating innate immune responses in CRC. We found that β -catenin negatively regulated the translocation of IRF3 into the nucleus and thus inhibited innate immune responses in CRC. This study expands our understanding on the crosstalk between an oncogene and innate immune responses, and may explain in part the enhanced ability to escape innate immunity in oncogenic transformed colorectal cells.

2 | MATERIALS AND METHODS

2.1 | Cell lines and viruses

HEK293T, BHK21, Vero, HCT116, DLD-1, Lim2405 and RKO were cultured in Dulbecco's modified Eagle's medium (DMEM, Corning), HT29 and Colo205 were cultured in Roswell Park Memorial Institute-1640 (RPMI1640, Corning), Lovo-p1 was cultured in Ham's F 12 nutrient medium (F12K, Corning), SW620 and SW480 were cultured in Leiboviz's L15(L15). All the mediums were supplemented with 10% heat-inactivated foetal bovine serum (FBS; HyClone), penicillin (100 U/mL) and streptomycin (100 μ g/mL). CRC cell lines, including SW480, SW620, DLD-1, Lim2045, RKO, HT29, Colo205, Lovo-p1, DiFi, were gifts from Dr. Chengyu Liang (USC). Vascular Stomatitis Virus (VSV-eGFP, provided by Dr. Sean Whelan) was amplified in BHK21 cells, with viral titre of 10^9 pfu per mL. Sendai virus (SeV) was purchased from Charles River Laboratories.

2.2 | Constructs

Luciferase reporter plasmids for the IFN- β promoter, expression plasmids for RIG-I-N, MAVS, TBK1 and IRF3-5D were described

previously (Dong et al., 2010; Dong and Feng, 2011; Dong et al., 2012; He et al., 2015; Seth et al., 2005). The non-silencing (control) shRNA and shRNA against human β -catenin were purchased from Thermo Scientific. Lentiviral expression plasmids for β -catenin were generated by standard molecular cloning techniques.

2.3 | Antibodies and reagents

Antibodies against FLAG (M2, Sigma), β -catenin (H102, Santa Cruz Biotechnology), IRF3 (FL-425, Santa Cruz Biotechnology), β -Actin (Ab8226, Abcam), α -Tubulin (11224-1-AP, PROTEINTECH), Phospho-IRF3 (Ser396) (RS-04 3195R, BIOSS) and Sendai virus (PD029, MBL) were purchased from the indicated suppliers.

2.4 | DNA transfection

For plasmid transfection in HEK293T cells, calcium phosphate transfection method was applied. HEK293T cells were plated at around 50%-60% confluence.

2.5 | Lentivirus-mediated stable cell line construction

HEK293T cells were transfected with the packaging plasmids VSV-G, DR8.9 and the lentiviral shRNA plasmids. At 48 hours post-transfection, supernatant was harvested and filtered (and concentrated by centrifugation if necessary). CRC cells were infected with the supernatant in the presence of polybrene (8 μ g/mL) by centrifugation at 1800 rpm (Beckman Coulter, Allegra X-15R) for 45 minutes. Cells were selected at 48 hours post-infection and maintained in 10% FBS DMEM supplemented with puromycin (1–2 μ g/mL).

2.6 | Immunohistochemistry analysis

A total of 60 paired tumour samples and matched para-cancerous tissue were collected from patients with CRC (median age 66 years, range 24–89, 33 male patients and 27 female patients) at Xiangya Hospital of Central South University from January to December of 2016. All patients were staged I, II or III (not distantly metastasized at the time point of surgery). They were previously untreated. CRC patient tissues were fixed in 10% neutral buffered formalin (Sigma) overnight at room temperature. Tissues were then dehydrated, embedded in paraffin and cut into 3 μ m sections. After antigen retrieval, tissue sections were subject to immunohistochemical staining with control antibodies or antibodies against β -catenin. CRC patients with informed consent donated their CRC tissues for the study and ethical approval was given by the medical ethics committee of Xiangya Hospital of Central South University with the following reference number: 201610067.

2.7 | Plaque assay

VSV titre was determined by plaque assay on Vero monolayer cells as previously described.¹² Briefly, 10-fold serially-diluted virus-containing supernatant was added into Vero cells and incubated for 2 hours at 37°C. Then, DMEM containing 2% FBS and 1% methylcellulose (Sigma) was added after removing the supernatant. Plaques were counted at day 3 post-infection.

2.8 | Quantitative Real-time PCR (qRT-PCR)

Quantitative Real-time PCR was performed as previously described.¹² Cells were infected or treated with viruses or agents respectively for the indicated time period. Total RNA was extracted using TRIzol reagent (Invitrogen). Complementary cDNA was synthesized from DNase I-treated total RNA using reverse transcriptase (Invitrogen). cDNA was diluted and qRT-PCR was performed using SYBR Green Master Mix (Applied Biosystems) by real-time PCR instrument (Applied Biosystems). Relative mRNA expression for each target gene was calculated by the $2^{-\Delta\Delta C_t}$ method using β -Actin as an internal control. The sequences of qRT-PCR primers are as follows:

Human β -actin	Forward	5'-CTGGCAGCCAGCACAATG-3'
	Reverse	5'-GCCGATCCACACGGAGTACT-3'
Human Ifnb	Forward	5'-AGGACAGGATGAACTTTGAC-3'
	Reverse	5'-TGATAGACATTAGCCAGGAG-3'
Human ISG56	Forward	5'-TCTCAGAGGAGCCTGGCTAA-3'
	Reverse	5'-TGACATCTCAATTGCTCCAG-3'

2.9 | Luciferase reporter assay

HEK293T cells, seeded in 24-well plates (~50% cell density), were transfected with IFN- β -Luc reporter plasmid cocktail (50 ng of luciferase reporter plasmid and 100 ng of β -galactosidase control vector) and β -catenin by calcium phosphate transfection. Whole cell lysates were used to determine the activity of firefly luciferase and β -galactosidase by a microplate reader (FLUOstar Omega).

2.10 | Immunoblotting

Cells were collected, rinsed with ice-cold PBS and lysed in NP-40 buffer (50 mM Tris-HCl [pH7.4], 150 mM NaCl, 1% NP-40, 5 mM EDTA) supplemented with a protease inhibitor cocktail (Roche) and sonicated for 3 times on ice. Whole cell lysates were denatured and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membrane and blocked in TBS-T containing 3% non-fat milk for 1 hour at room temperature. Membrane was incubated with primary antibodies (1:1000 dilution) overnight at 4°C. Blots were washed extensively and incubated with corresponding IRDye800 conjugated

secondary antibodies (Licor) at 1:10000 for one hour at room temperature. Proteins were visualized using Odyssey infrared imaging system (Licor).

2.11 | Co-immunoprecipitation (Co-IP) and immunoblotting

For immunoprecipitation, cells were harvested and whole cell lysates were prepared with NP-40 buffer supplemented with 20 mM β -glycerophosphate and 1 mM sodium orthovanadate. Then, whole cell lysates were sonicated, centrifuged and pre-cleared with Sepharose 4B agarose beads for 1 hour. Pre-cleared samples were then incubated with the indicated antibodies and protein A/G agarose (Thermo Scientific), antibody-conjugated agarose (Sigma), or glutathione agarose beads (GE Healthcare) at 4°C for 4 hour. The agarose beads were washed 3 times with lysis buffer and eluted with 1 $\times$ SDS sample buffer by boiling at 95°C for 10 minutes. Precipitated proteins were analysed by SDS gel electrophoresis and immunoblotting.

2.12 | Nucleus-cytoplasm extraction

SW620 cells stably knocking down of β -catenin were infected with Sendai virus for 12 hours (100 [HAU]/mL). Cells were harvested, washed by ice-cold PBS, micro-centrifuged at 1000 rpms (ependorf centrifuge 5424), re-suspended with cytoplasmic extract (CE) buffer (10 mM HEPES, 60 mM KCl, 1 mM EDTA, 1 mM DTT and 1 mM PMSF, adjusted to pH 7.6) with 0.075% (v/v) NP-40, and incubated on ice for 5 minutes. Samples were then centrifuged and supernatant was collected as the cytoplasmic extract. The pellet (nuclei) with cytoplasmic extract (CE) buffer without NP-40, and then resuspended in nuclear extract (NE) buffer (20 mM Tris Cl, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM PMSF and 25% (v/v) glycerol, adjusted to pH 8.0). The cytoplasmic and nuclear extractions were analysed by SDS gel electrophoresis and immunoblotting.

2.13 | Confocal microscopy

DLD-1 cells stably knocking down of β -catenin were infected with SeV for 12 hours (100 [HAU]/mL). Cells were fixed on slides, permeabilized and stained with indicated primary antibody (1:100). The slides were then stained with Alexa Fluor 488/594-conjugated goat secondary antibody (1:200), and analysed by confocal microscope (Leica). Representative images were shown for all analyses.

2.14 | Statistical analysis

Statistical analysis was performed by unpaired two-tailed Student's *t* test. A *P*-value less than .05 is considered statistically significant.

2.15 | Abbreviation list

Abbreviation	Full name
CRC	Colorectal cancer
IFN- β	Interferon- β
RIR	Rig-I-like receptor
IRF3	Interferon-regulatory factor 3
ISG56	Interferon-stimulated gene 56
NF- κ B	Nuclear factor κ B
TGF- β	Transforming growth factor- β
PRRs	Pattern-recognition receptors
DAMPs	Damage-associated molecular patterns
TLRs	Toll-like receptors
cGAS	Cyclic GMP-AMP synthase
DAI	DNA-dependent activator of IFN-regulatory factors
DMEM	Dulbecco's modified Eagle's medium
RPMI1640	Roswell park memorial institute-1640
F12K	Ham's F 12 nutrient medium
L15	Leiboviz's L15
FBS	Foetal bovine serum
SeV	Sendai virus
RIG-I	Retinoic acid-inducible gene I
RIG-I-N	Rig-I N-terminal tandem CARD
TBK1	Tank-binding kinase 1
MAVS	Mitochondrial antiviral signalling protein
rpm	Revolutions per minute
VSV	Vesicular Stomatitis Virus
EDTA	Ethylenediaminetetraacetic acid
SDS	Sodium dodecyl sulphate
TBS-T	Triethanolamine buffered saline solution- tween
Co-IP	Co-immunoprecipitation
CE	Cytoplasmic extract
DTT	DL-Dithiothreitol
PMSF	Phenylmethanesulfonyl fluoride
NE	Nuclear extract
IHC	Immunohistochemistry
CARD	Caspase activation and recruitment domains

3 | RESULTS

3.1 | The expression of β -catenin is positively correlated with IRF3 in CRC

Aberrant expression of β -catenin is one of the hallmarks in CRC. As expected, immunohistochemistry (IHC) staining demonstrated that β -catenin, IRF3 and p-IRF3 were highly expressed in CRC patient samples (Figure 1A). The results were consistent with other studies in the tumorigenesis of CRC.²⁴

FIGURE 1 The high expression of β -catenin is positively correlated with the expression of IRF3 in CRC. (A) Immunohistochemistry staining of β -catenin, IRF3, phospho-IRF3 (p-IRF3) in human CRC tissue and para-cancerous tissue. (B) Whole cell lysates were extracted from different CRC cell lines and analysed by immunoblotting with indicated antibodies. (C) Whole cell lysates were extracted from different CRC cell lines and analysed by immunoblotting with indicated antibodies

As tumour-associated RNA/DNA leaking or necrotic cell death will cause the release of DAMP molecules, we hypothesize that in CRC cells, DAMPs induced, IRF3-mediated type I interferon signalling is likely to be activated during tumourigenesis. As shown in Figure 1A, the phosphorylation of IRF3 (an indication of activated interferon signalling) was highly expressed in CRC patient samples. The results were consolidated because we detected the phosphorylation of IRF3 in some of CRC cell lines (Figure 1B). The data indicated that the RIG-I-like receptor (RLR)-mediated IFN- β signalling pathway in CRC was activated.

We then assessed the expression of β -catenin, the key type I interferon transcription factor IRF3 in different CRC cell lines. The overall expression of β -catenin was high and positively correlated with IRF3 in most of the cell lines (Figure 1C). These data prompted us to investigate the correlation between β -catenin and IRF3-mediated innate immune signalling during colorectal tumourigenesis.

3.2 | β -catenin disrupts antiviral response in CRC cells

To probe the potential role of β -catenin in IRF3-mediated innate immunity, we used GFP-tagged VSV infection to assess its effect on

antiviral responses. Exogenous overexpression of β -catenin in two CRC cell lines HCT116 and DLD-1 increased viral titres of VSV by about 3-folds (Figure 2A,B). In contrast, knocking down of β -catenin in the cell lines of SW620 and HT29 (another 2 CRC cells lines with relatively high β -catenin protein level) markedly reduced the titres of VSV (Figure 2C). The decrease in the replication of VSV was further confirmed by GFP intensity, indicating that β -catenin-deficient cells were more resistant to VSV infection than the control group (Figure 2D,E). Taken together, these results suggested that β -catenin abrogated the antiviral activity of CRC cells.

3.3 | β -catenin negatively regulates IFN- β production in CRC cells

Interferon beta (IFN- β) signalling, as the major type I interferon signalling, plays utmost roles in the antiviral responses against VSV.²⁵ Thus we investigated whether β -catenin inhibited the transcription of IFN- β gene upon viral infection. Indeed, knocking down of β -catenin in HCT116, DLD-1 and SW620 cell lines significantly up-regulated the expression of IFN- β induced by SeV (Figure 3A). When probing for the expression of the interferon-stimulated genes (ISGs), we found that the expression

FIGURE 2 β -catenin disrupts antiviral response in CRC cells. (A) HCT116 cells stably expressing control (NC), β -catenin were prepared by lentiviral transduction. Cells were then infected with VSV-eGFP (MOI = 0.1) for the indicated hours. The supernatant was harvested and VSV-eGFP viral titre was measured by plaque assay. $*P < .01$. (B) DLD-1 cells stably expressing control (NC), β -catenin were prepared by lentiviral transduction. Cells were then infected with VSV-eGFP (MOI = 0.1) for the indicated hours. The supernatant was harvested and VSV-eGFP viral titre was measured by plaque assay. $**P < .05$. (C) SW620 cells stably expressing control (shNC), β -catenin shRNA (sh β -catenin) were prepared by lentiviral transduction. Cells were then infected with VSV-eGFP (MOI = 0.1) for the indicated hours. The supernatant was harvested and VSV-eGFP viral titre was measured by plaque assay. $*P < .01$. (D) HT29 cells stably expressing control (shNC), β -catenin shRNA (sh β -catenin) were prepared by lentiviral transduction. Cells were then infected with VSV-eGFP (MOI = 0.1) for the indicated hours. GFP of cells was checked by microscope. (E) SW620 and HT29 cells stably expressing control (shNC), β -catenin shRNA (sh β -catenin) were prepared by lentiviral transduction. Cells were then infected with VSV-eGFP (MOI = 0.1) for the indicated hours. GFP of cells was checked by microscope. For (A–C) data are presented as mean $\pm$ SD. Independent experiments were repeated 2 or 3 times

of ISG56 was also significantly increased in SW620 cells upon β -catenin depletion (Figure 3B). These results clearly demonstrated a negative role of β -catenin in regulation of type I interferon signalling in CRC cells.

3.4 | β -catenin inhibits RIG-I-like receptor (RLR)-mediated IFN- β signalling pathway in CRC cells

It is well-known that both SeV and VSV are critical inducers of the RIG-I-like receptor (RLR)-mediated IFN- β signalling pathway.²⁵ As shown in the diagram illustrates the RIG-I-IFN- β signalling pathway (Figure 4A), we reasoned that β -catenin ought to inhibit one of the signalling proteins along the RIG-I-IFN- β signalling axis. To identify the target protein, we examined the effect of β -catenin on the IFN- β transcription activity induced by every single RIG-I signalling proteins including RIG-I-2CARD (the 2 caspase activation and recruitment domains of RIG-I which is constitutively active), MAVS, TBK1 and IRF3-5D (a constitutively active IRF3).²⁶ Interestingly, we found that the activity of the IFN- β reporter was reduced upon overexpression of β -catenin in all the experiment

settings, including RIG-I-2CARD, MAVS, TBK1 and IRF3-5D (Figure 4B,C). This demonstrated that β -catenin directly targeted the most downstream protein of the RIG-I signalling axis, which was IRF3. To consolidate our finding, we sought out to study the interaction between IRF3 and β -catenin. As shown in co-immunoprecipitation (Co-IP) assay, IRF3 was readily pulled down by exogenously expressed β -catenin in 293T cells (Figure 4D) and the endogenous β -catenin in HCT116. Furthermore, the interaction between IRF3 and β -catenin was increased upon SeV infection (Figure 4E). These results indicated that β -catenin directly interacted with and exerted an inhibitory effect on IRF3, which explained its negative role in the innate immunity and antiviral responses in CRC cells.

3.5 | β -catenin blocks the nuclear translocation of IRF3 in CRC cells

Finally, we studied the possible mechanism(s) of β -catenin's inhibition on IRF3. IRF3 nuclear translocation represents one of the key

FIGURE 3 β -catenin negatively regulates IFN- β production in CRC cells. (A) HCT116, DLD-1 and SW620 cells stably expressing control (shNC), β -catenin shRNA (sh β -catenin) were prepared by lentiviral transduction. Cells were then infected with SeV (100 [HAU]/mL) for the indicated hours. RNA was extracted and cDNA was prepared to determine IFN- β mRNA expression by real-time PCR. (B) SW620 cells stably expressing control (shNC), β -catenin shRNA (sh β -catenin) were prepared by lentiviral transduction. Cells were then infected with SeV (100 [HAU]/mL) for the indicated hours. RNA was extracted and cDNA was prepared to determine ISG56 mRNA expression by real-time PCR. For (A-B) data are presented as mean $\pm$ SD. Independent experiments were repeated 2 or 3 times. * P < .01

steps for IFN- β transactivation.^{21,22} As β -catenin binds to IRF3, we were wondering whether β -catenin may interfere with IRF3 nuclear translocation upon type I innate immune signalling activation. As analysed by cell fractionation, knocking down of β -catenin in SW620 cells significantly increased the nuclear portion of IRF3 induced by SeV (Figure 5A,B), and overexpressing β -catenin in HCT116 cells significantly inhibited the nuclear portion of IRF3 induced by SeV (Figure 5C). To validate this finding, immunofluorescence staining of IRF3 was performed upon knocking down of β -catenin in another SeV-induced CRC cell line, DLD-1. Indeed, β -catenin depletion significantly resulted in the accumulation of IRF3 in the nucleus (Figure 5D,E). Collectively, our data supported the hypothesis that β -catenin inhibited the IRF3-mediated immune signalling pathway by blocking the nuclear translocation of IRF3 in CRC cells. This finding added a novel tumorigenic role of β -catenin in CRC progression which is the evasion of innate immunity. Meanwhile, our research is of clinical relevance as it provided hints for a potential synergistic effect of treating CRC with WNT/ β -catenin signalling inhibitors and immunotherapy methods such as CAR-T.

4 | DISCUSSION

For malignant tumour to survive and progress in immunocompetent individuals, tumours cells must develop strategies to evade the immunosurveillance. Some of these strategies include overexpression of immunosuppressive molecules (eg, TGF- β , IL-10, PD-L1, TIM3, TIGIT and prostaglandins).²⁷⁻³⁰ These molecules will enable the escape of tumour cells from immune-mediated

killing, and the prevention of the release of tumour cell surface antigens.³¹ On the other hand, how tumour cells escape the innate immunity, which recognizes tumour-associated molecular patterns and primes the adaptive immune responses, remains poorly understood.

β -catenin, as proto-oncogene, has been studied extensively since the discovery of its aberrant various human cancers.^{32,33} Here, we report a novel function of β -catenin, which is the inhibition of type I interferon signalling in CRC cells. We propose that β -catenin contributes to the tumour evasion of host recognition and type I innate immune activation. Mechanistically, we demonstrated that β -catenin targets IRF3, the critical transcription factor for IFN- β production. Our study uncovered a novel crosstalk between tumour oncogene and the IRF3-mediated innate immune responses, providing another immunotherapy strategy to boost host immune defence against tumour progression by targeting β -catenin.

From our studies, we found that β -catenin blocked IFN- β production and supported VSV replication. This finding is not unexpected, as β -catenin is the main components of cell cytoskeleton. Ample evidences have shown that most of components of cytoskeleton, including microfilaments, F-actin, microtubules and intermediate filaments can help virus to evade immunosurveillance of host and benefit to virus assembly and budding.³⁴ We also noticed that in another paper, the authors found high level of β -catenin could inhibit viral replication in lung epithelial cells by enhancing the virus-dependent induction of the IFNB1 gene and interferon-stimulated genes.³⁵ We think that the contradictory findings may due to the difference between normal cells and CRC cells. Mutations and overexpression of β -catenin are commonly

FIGURE 4 β -catenin inhibits RIG-I-like receptor (RLR)-mediated IFN- β signalling pathway in CRC cells. (A) Diagram illustrates the RLR IFN- β signalling pathway leading from PRRs to activation of the IFN- β gene transcribed by IRF3: PRRs can detect and recognize molecules typical for pathogens, such as 5'-triphosphate RNA from virus. RNA binds to and activates RIG-I-MAVS-TBK1/IKK ϵ signalling cascade, which can phosphorylate transcription factor IRF3. Phosphorylated IRF3 then translates into nucleus to turn on the expression of genes of IFNs and ISGs. (B) HEK293T cells were transfected with empty vector or vector containing a plasmid encoding Flag-tagged RIG-I (amino terminus (RIG-I-N); amino acids 1-200), MAVS or TBK1 (bottom), increasing amount of β -catenin and the same amount of IFN- β luciferase reporter cocktail for 30 hours. Activation of RIG-I signalling was determined by luciferase reporter assay. * $P < .01$. (C) HEK293T cells were transfected with empty vector or vector containing a plasmid encoding IRF3-5D (bottom), increasing amount of β -catenin and the same amount of IFN- β luciferase reporter cocktail for 30 hours. Activation of RIG-I signalling was determined by luciferase reporter assay. * $P < .01$. (D) HEK293T cells were transfected with plasmids containing β -catenin and IRF3. Whole cell lysates were precipitated with the indicated antibody. Whole cell lysates and precipitated proteins were analysed by immunoblotting with indicated antibodies. (E) HCT116 cells stably expressing control (shNC), β -catenin shRNA (sh β -catenin) were prepared by lentiviral transduction. Cells were then infected with mock or SeV (100 [HAU]/mL) for the indicated hours. Whole cell lysates were precipitated with the indicated antibody. Whole cell lysates and precipitated proteins were analysed by immunoblotting with indicated antibodies. Independent experiments were repeated 2 or 3 times

found in a variety of cancers, especially in CRC. In physiological condition, regular levels of β -catenin with no mutations may assist cells to resist various harmful microbes, including virus via acting in concert with the p300 transcription co-factor and binds the IFN- β promoter to increase the production of IFN- β . But in cancer cells the overexpressed and mutated β -catenin will have different or even contrary function on IRF3 signalling.

It is widely acknowledged that IRF3 is the key transcription factor mainly responsible for the production of IFN- β . The activation of IRF3 contains several essential steps: phosphorylation, homodimerization and nuclear translocation. We found that β -catenin interacted with IRF3 and blocked its nuclear translocation. The binding

of β -catenin to IRF3 is also indirectly supported by other studies, in which cytoskeleton microtubules can interact with IRF3.³⁶ The mechanism beyond this inhibition could be explained by either direct trapping IRF3 in the cytoplasm or blocking its association with TBK1, resulting in failure of phosphorylation and dimerization. Future studies will be needed to characterize the nature of it.

β -catenin is one of commonly mutated or overexpressed oncogenes in CRC.^{32,33} We demonstrated these "high β -catenin" CRC cells had "crippled" production of type I interferon and antiviral responses, and some virus infection can induce the occurrence and development of CRC by enhances CRC cells proliferation, migration and upregulation of EMT markers,³⁷ thus they might represent

FIGURE 5 β-catenin blocks the nuclear translocation of IRF3 in CRC cells. (A) SW620 cells stably expressing control (shNC), β-catenin shRNA (shβ-catenin) were prepared by lentiviral transduction. Cells were then infected with mock, SeV (100 [HAU]/mL) for the indicated hours. Whole cell lysates (WCL) were analysed by immunoblotting with indicated antibodies. (B) Prepared cells as in (A), then followed by nucleus-cytoplasm extraction. Normalized to those of histone and β-actin. (C) HCT116 cells stably expressing control (NC), β-catenin-Flag were prepared by transfection. Cells were then infected with mock, SeV (100 [HAU]/mL) for the indicated hours. Whole cell lysates (WCL), nucleus extraction (N), cytoplasm extraction (C) were analysed by immunoblotting with indicated antibodies. Normalized to those of histone and α-tubulin. (D) DLD-1 cells stably expressing control (shNC), β-catenin shRNA (shβ-catenin) were prepared by lentiviral transduction. Cells were then infected with SeV (100 [HAU]/mL) for the indicated hours. IRF3 nuclear translocation was analysed by immunofluorescence microscopy with anti-IRF3 antibody. (E) The percentage of IRF3 nuclear translocation was calculated based on the immunofluorescence result by ImageJ Software, * $P < .01$ (Student's t test). Independent experiments were repeated 2 or 3 times

optimal targets for interferon-sensitive oncolytic viruses. Our results also demonstrated that down regulation of β-catenin in CRC cells led to the increase of the production of type I interferon production and led to reduce the susceptibility to VSV. Therefore, our work provides a theoretical basis for the screening and clinical treatment

of β-catenin related tumours such as CRC by oncolytic viruses such as VSV.

Type I interferons have multiple potential functions on tumour cells, including the promotion of apoptosis, inhibition of proliferation and tumour angiogenesis and activation of innate and adaptive

immune responses.^{38,39} We speculate that ablation of the production of type I interferon by β -catenin in CRC might further contribute to tumour development. Overall, we consider that β -catenin might exert oncogene activity in part through inhibition of innate immune responses via down regulation of the production of type I interferon in CRC, for its escape of host innate immunity defence. Additionally, we have expanded β -catenin's repertoire as an oncogene via antagonizing RIG-I-like receptor (RLR)-mediated IFN- β signalling pathway. Taken together, our findings have revealed the novel function of β -catenin in innate immunity which will benefit the development of efficient immune therapeutic interventions and oncolytic treatment for human CRC.

ACKNOWLEDGEMENTS

This work was supported by the National Key Research and Development Program of China (2016YFC1200200 and 2016YFD0500300) (to Z.X.), the Joint Funds of the National Natural Science Foundation of China (U1603126) (to Z.X.), the Hunan Provincial Natural Science Fund (2017JJ2334) (to J.P.), the "555 Talent Program" in Changsha National High-tech Industry Development Zone (to Z.X.), the Hunan Provincial Natural Science Fund (2018JJ3471) (to C.D.), the Research Project of Hunan Health and Family Planning Commission (C20180156) (to C.D.), and the Project of China Scholarship Council (201406370025) (to C.D.). The authors are grateful to Dr. Pinghui Feng and Dr. Chengyu Liang (University of Southern California).

ORCID

Chengming Ding <http://orcid.org/0000-0002-6979-1221>

Jian Peng <http://orcid.org/0000-0003-0991-3896>

REFERENCES

- Morin PJ. β -catenin signaling and cancer. *BioEssays*. 1999;21:1021-1030.
- Subramanian M, Rao SR, Thacker P, Chatterjee S, Karunakaran D. MiR-29b downregulates canonical Wnt signaling by suppressing coactivators of β -catenin in human colorectal cancer cells. *J Cell Biochem*. 2014;15:1974-1984.
- Tanksley JP, Chen X, Coffey RJ. NEDD4L is downregulated in colorectal cancer and inhibits canonical WNT signaling. *PLoS ONE*. 2013;8:e81514.
- Barker N, Clevers H. Catenins, Wnt signaling and cancer. *BioEssays*. 2000;22:961-965.
- Deng J, Miller SA, Wang H-Y, et al. β -catenin interacts with and inhibits NF- κ B in human colon and breast cancer. *Cancer Cell*. 2002;2:323-3234.
- Ke B, Shen XD, Kamo N, et al. β -catenin regulates innate and adaptive immunity in mouse liver ischemia-reperfusion injury. *Hepatology*. 2013;57:1203-1214.
- Ranoa DR, Parekh AD, Pitroda SP, et al. Cancer therapies activate RIG-I-like receptor pathway through endogenous non-coding RNAs. *Oncotarget*. 2016;7:26496-26515.
- Thaiss CA, Zmora N, Levy M, Elinav E. The microbiome and innate immunity. *Nature*. 2016;535:65-74.
- Gajewski TF, Schreiber H, Fu Y-X. Innate and adaptive immune cells in the tumor microenvironment. *Nat Immunol*. 2013;14:1014-1022.
- Croci DO, Zacarias Fluck MF, Rico MJ, Matar P, Rabinovich GA, Scharovsky OG. Dynamic cross-talk between tumor and immune cells in orchestrating the immunosuppressive network at the tumor microenvironment. *Cancer Immunol Immunother*. 2007;56:1687-1700.
- Pang IK, Ichinohe T, Iwasaki A. IL-1R signaling in dendritic cells replaces pattern-recognition receptors in promoting CD8⁺ T cell responses to influenza A virus. *Nat Immunol*. 2013;14:246-253.
- Zhao Jun, Zeng Yi, Simin Xu, et al. A viral deamidase targets the helicase domain of RIG-I to block RNA-induced activation. *Cell Host Microbe*. 2016;20:770-784.
- Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol*. 2010;11:373-384.
- Wang H, Hu S, Chen X, et al. cGAS is essential for the antitumor effect of immune checkpoint blockade. *Proc Natl Acad Sci USA*. 2017;114:1637-1642.
- Chen Q, Sun L, Chen ZJ. Regulation and function of the cGAS-STING pathway of cytosolic DNA sensing. *Nat Immunol*. 2016;17:1142-1149.
- Llader A, Mougialakos D, Tufvesson H, et al. DAI (DLM-1/ZBP1) as a genetic adjuvant for DNA vaccines that promotes effective antitumor CTL immunity. *Mol Ther*. 2011;19:594-601.
- Xu RH, Wong EB, Rubio D, et al. Sequential activation of two pathogen-sensing pathways required for type I interferon expression and resistance to an acute DNA virus infection. *Immunity*. 2015;43:1148-1159.
- Fondello C, Agnetti L, Villaverde MS, Simian M, Glikin GC, Finocchiaro LM. The combination of bleomycin with suicide or interferon- β gene transfer is able to efficiently eliminate human melanoma tumor initiating cells. *Biomed Pharmacother*. 2016;83:290-301.
- Sakisaka M, Haruta M, Komohara Y, et al. Therapy of primary and metastatic liver cancer by human iPS cell-derived myeloid cells producing interferon- β . *J Hepatobiliary Pancreat Sci*. 2017;24:109-119.
- Yoon N, Park MS, Shigemoto T, Peltier G, Lee RH. Activated human mesenchymal stem/stromal cells suppress metastatic features of MDA-MB-231 cells by secreting IFN- β . *Cell Death Dis*. 2016;7:e2191.
- Hiscott J, Pitha P, Genin P, et al. Triggering the interferon response: the role of IRF-3 transcription factor. *J Interferon Cytokine Res*. 1999;19:1-13.
- Au WC, Moore PA, Lowther W, Juang YT, Pitha PM. Identification of a member of the interferon regulatory factor family that binds to the interferon-stimulated response element and activates expression of interferon-induced genes. *Proc Natl Acad Sci USA*. 1995;92:11657-11661.
- Lefort S, Soucy-Faulkner A, Grandvaux N, Flamand L. Binding of Kaposi's sarcoma-associated herpesvirus K-bZIP to interferon-responsive factor 3 elements modulates antiviral gene expression. *J Virol*. 2007;81:10950-10960.
- Firestein R, Bass AJ, Kim SY, et al. CDK8 is a colorectal cancer oncogene that regulates β -catenin activity. *Nature*. 2008;455:547-551.
- Meylan E, Curran J, Hofmann K, et al. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature*. 2005;437:1167-1172.
- Lin R, Heylbroeck C, Pitha PM, Hiscott J. Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear

- translocation, transactivation potential, and proteasome-mediated degradation. *Mol Cell Biol.* 1998;18:2986-2996.
27. Han J, Chen X, Chu J, et al. TGF β treatment enhances glioblastoma virotherapy by inhibiting the innate immune response. *Cancer Res.* 2015;75:5273-5282.
 28. Mannino MH1, Zhu Z, Xiao H, Bai Q, Wakefield MR, Fang Y. The paradoxical role of IL-10 in immunity and cancer. *Cancer Lett.* 2015;367:103-107.
 29. Basingab FS, Ahmadi M, Morgan DJ. IFN γ -dependent interactions between ICAM-1 and LFA-1 counteract prostaglandin E2-mediated inhibition of antitumor CTL responses. *Cancer Immunol Res.* 2016;4:400-411.
 30. Neeman E1, Zmora O, Ben-Eliyahu S. A new approach to reducing postsurgical cancer recurrence: perioperative targeting of catecholamines and prostaglandins. *Clin Cancer Res.* 2012;18:4895-4902.
 31. Lafitte M, Rousseau B, Moranvillier I, et al. In vivo gene transfer targeting in pancreatic adenocarcinoma with cell surface antigens. *Mol Cancer.* 2012;11:81.
 32. Rosenbluh J, Nijhawan D, Cox AG, et al. β -Catenin-driven cancers require a YAP1 transcriptional complex for survival and tumorigenesis. *Cell.* 2012;151:1457-1473.
 33. Clevers H, Nusse R. Wnt/ β -catenin signaling and disease. *Cell.* 2012;149:1192-1205.
 34. Shahriari S, Gordon J, Ghildyal R. Host cytoskeleton in respiratory syncytial virus assembly and budding. *Virology.* 2016;13:161.
 35. Hillesheim A, Nordhoff C, Boergeling Y, Ludwig S, Wixler V. β -catenin promotes the type I IFN synthesis and the IFN-dependent signaling response but is suppressed by influenza A virus-induced RIG-I/NF- κ B signaling. *Cell Commun Signal.* 2014;12:29.
 36. Chiang HS, Zhao Y, Song JH, et al. GEF-H1 controls microtubule-dependent sensing of nucleic acids for antiviral host defenses. *Nat Immunol.* 2014;15:63-71.
 37. Teo WH, Chen H-P, Huang JC, Chan Y-J. Human cytomegalovirus infection enhances cell proliferation, migration and upregulation of EMT markers in colorectal cancer-derived stem cell-like cells. *Int J Oncol.* 2017;51:1415-1426.
 38. Shou P, Chen Q, Jiang J, et al. Type I interferons exert anti-tumor effect via reversing immunosuppression mediated by mesenchymal stromal cells. *Oncogene.* 2016;35:5953-5962.
 39. Zitvogel L, Galluzzi L, Kepp O, Smyth MJ, Kroemer G. Type I interferons in anticancer immunity. *Nat Rev Immunol.* 2015;15:405-414.

How to cite this article: Ding C, He J, Zhao J, et al. β -catenin regulates IRF3-mediated innate immune signalling in colorectal cancer. *Cell Prolif.* 2018;51:e12464. <https://doi.org/10.1111/cpr.12464>