

## Original Article

# MiR-214 inhibits cell proliferation and cell cycle through targeting wnt/ $\beta$ -catenin pathway in vitro and in vivo

Yumei Cai<sup>1,2\*</sup>, Ruixue Cui<sup>1,2\*</sup>, Jie Zhao<sup>1,2</sup>, Huaqing Wang<sup>1,2</sup>, Yu Cui<sup>1,2</sup>, Jingxiu Niu<sup>1,2</sup>, Feng Hua<sup>3</sup>, Lihong Zhang<sup>1,2</sup>, Qiang Yao<sup>1,2</sup>

<sup>1</sup>Department of Oncology, Tianjin Union Medicine Center, Tianjin 300121, China; <sup>2</sup>Tianjin Institute of Chinese Integrative Medicine of Oncology, Tianjin 300121, China; <sup>3</sup>Shandong Cancer Hospital, Jinan 250117, Shandong Province, China. \*Equal contributors.

Received September 2, 2016; Accepted October 14, 2016; Epub January 15, 2017; Published January 30, 2017

**Abstract:** Objectives: The malignancy of colon cancer (CC) is largely due to its infinite proliferation and strong survival, in which Wnt/ $\beta$ -catenin signaling plays a critical role. The aim of this study was to investigate the role and internal mechanism of microRNA-214 by targeting Wnt/ $\beta$ -catenin pathway in CC. Methods: We performed quantitative real time PCR (qPCR) to realize the expression level of miR-214 in selected CC samples and cell lines. After it was transiently forced expressed and/or knocked down by mimic/inhibitor transfections, cell proliferation and viability were accessed by CCK-8 and crystal violet staining. Luciferase reporter, qPCR and western blot assays were all carried out to investigate the impact of miR-214 on the Wnt/ $\beta$ -catenin signaling in vitro. Afterwards, tumor formation was done in a xerograph model. Tumor weight and volume were recorded and expressions of related molecules were analyzed. Results: CC samples and cell lines had significantly lower level of miR-214. Overexpression of miR-214 inhibited cell growth and viability, while its deletion promoted CC cell growth and viability *in vitro*. We further found that Wnt/ $\beta$ -catenin signaling could be negatively regulated by miR-214. Additionally, our *in vivo* data suggested an anti-tumor role of miR-214 in CC. Conclusion: Our data demonstrated a previously unappreciated role for miR-214 in suppression of Wnt/ $\beta$ -catenin-mediated CC cell growth, and highlight miR-214 as a potent suppressor of CC pathology.

**Keywords:** MiR-214, cell growth and viability, Wnt/ $\beta$ -catenin, colon cancer

## Introduction

MicroRNAs (miRNAs) are a class of small, non-coding RNAs that control diverse biological processes by post-transcriptional regulation. As pivotal post-transcriptional gene expression regulators, miRNAs induce transcript degradation or translational inhibition through their base-pairing to the 3'-untranslated region (3'-UTR) of target mRNAs [1-3]. It has been well acknowledged that miRNAs play a critical role during carcinogenesis of various cancers, by influencing various critical biological events, including the proliferation, differentiation, apoptosis and metastasis of tumor cells [4, 5]. Therefore, a better understanding of miRNA biology might ultimately yield further insights into the molecular mechanisms of tumorigenesis and new therapeutic strategies against cancer.

Recently, deregulated miR-214 has been shown to be associated with a variety of cancer types [6-8]. However, according to previous reports, miR-214 plays differential roles, which may even be opposing roles, in different types of cancer. For instance, miR-214 was found to be increased and contributed to disease progression and distant metastasis of malignant melanoma [9, 10]. By contrast, miR-214 is down-regulated and acts as a tumor suppressor, by reducing hepatoma cancer cell growth, metastasis and tumor angiogenesis of hepatoma [7, 11]. In colon cancer (CC), only two groups had reported the expression and role of miR-214. One group, miR-214 was demonstrated to be downregulated in CC tissues compared to healthy colon tissues; its overexpression results in the inhibition of cell viability, colony formation and proliferation, and induction of cell apoptosis, via the suppression of ADP-ribosylation

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factor-like protein 2 (ARL2) [12]. Another group, through identifying miRNAs involved in the pathology of CC liver metastasis, they validated that miR-214 was significantly downregulated in CC with liver metastasis, which was associated with an unfavorable prognosis. Also, miR-214 could suppress proliferation, migration, and invasion *in vitro*, tumor growth and liver metastasis *in vivo* by at least targeting of fibroblast growth factor receptor 1 (FGFR1) [13]. However, the role of miR-214 in CC still requires more investigation as the underlying mechanisms of action remain poorly understood. In another word, there might be other signaling networks regulated by miR-214 in CC cells.

In the present study, the expression pattern of miR-214 in CC samples and cell lines were determined. Importantly, we examined the effects of miR-214 on cell growth and viability both *in vitro* and *in vivo*. Furthermore, the underlying mechanisms of action of miR-214, through repressing Wnt/ $\beta$ -catenin signaling were also intensively explored.

### Materials and methods

#### *Patients & tissues*

25 colon cancer samples and their adjacent non-cancerous samples were collected from patients in Tianjin Union Medical Center from 2015-2016. The research was approved by the Ethics Committee of Tianjin Union Medical Center. Written informed consents were obtained from all of the patients. All paired samples were immediately frozen by liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until used.

#### *Cell lines and reagents*

Human fetal colon epithelial FHC cells and colon cancer HCT116, LS174T, Caco-2, SW480, SW620, LoVo and RKO cells were cultured in certain medium with 10% FBS, penicillin and streptomycin. The CCND1, CMYC and  $\alpha$ -tubulin antibodies were purchased from Cell Signaling Technology.

#### *RNA isolation and quantitative real-time PCR*

Total RNAs were isolated by TRIzol reagent from Invitrogen as per the manufacturer's protocol. The isolated RNA was reverse-transcribed into gene cDNAs or miR-214 cDNAs by reverse transcription kit from Promega by

using oligo(dT)<sub>n</sub> primer or miR-214 specific transcriptional primer. Quantitative real-time PCR (qPCR) was performed by SYBR Green Realtime PCR Master Mix from TOYOBO, and data collection was performed on an ABI7500 system. The results were normalized against  $\alpha$ -tubulin expression for genes and U6B expression for microRNAs. All the qPCR reactions were performed in triplicate.

#### *Mimics, inhibitors and transfections*

MiR-214 mimics, inhibitors and control oligoes were purchased from GenePharma. Transfections were performed by Lipofectamine® RN-AiMAX according to the manufacturer's instructions.

#### *CCK-8 assay*

HCT116 or LoVo cells transfected with miR-214 mimics or inhibitors with control oligoes, were trypsinized and seeded into 96-well plates at a density of 3000 cells in 200  $\mu\text{l}$  of medium per well and then incubated at  $37^{\circ}\text{C}$ . CCK-8 assay was performed by CCK-8 Kit from DOJINDO for continuous four days. Absorbance was detected at 590 nm using a microplate reader.

#### *Crystal violet staining*

The viability of HCT116 and LoVo cells was determined by crystal violet staining. The transfected cells grown in 6-well plates were fixed in 4% paraformaldehyde for 0.5 h. After washing twice, the cells were stained with 0.1% crystal violet for 0.5 h. The plates were aspirated, washed twice and allowed to air dry, followed by photographed in digital camera.

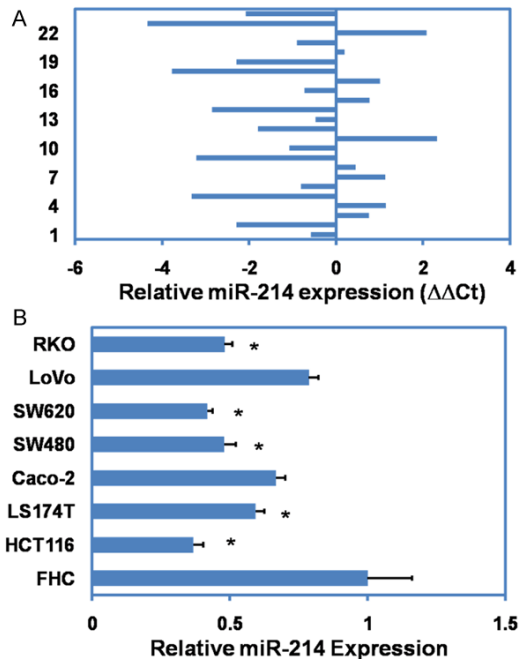
#### *Luciferase reporter assay*

Cells were plated at a subconfluent density and cotransfected with 0.5  $\mu\text{g}$  of the TOP flash or FOP flash reporter plasmid, miR-214 mimics or inhibitors and 0.02  $\mu\text{g}$  of renilla reporter plasmid as an internal control for transfection efficiency. Cell lysates were prepared 48 h after transfection, and the reporter activity was measured using the Luciferase Reporter Assay System from Promega.

#### *Western blot*

HCT116 or LoVo Cells were lysed in protein RIPA buffer and were cleared by centrifugation

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**Figure 1.** Reduced expression of miR-214 in CC samples and cell lines. A. The expression levels of miR-214 in 25 CC samples and adjacent non-cancerous samples were assessed by qPCR. The relative miRNA expression levels are presented as fold change =  $2^{-\Delta\Delta Ct}$  of tumor samples versus matched non-cancerous samples. B. The expression levels of miR-214 in human fetal colon epithelial FHC cells and seven CC cell lines were assessed by qPCR; \* $P < 0.05$ , compared with FHC.

at 12000 rpm for 40 min at 4°C. Protein concentration was measured by the BCA Protein Assay Kit from Beyotime. Proteins were denatured at 100°C for 10 min prior to loading onto 8% SDS-PAGE gel. After separation, proteins were transferred to NC membranes and incubated with appropriate primary antibodies at 4°C over night. The membranes were washed four times for each 5 min and incubated with HRP-conjugated secondary antibodies for 1.5 h at RT, followed by another three washing steps. Proteins were visualized by chemiluminescence detection imaging.

### Lenti-Virus particles

MiR-214 expressing lenti-virus particles and control virus (LV-NC-mi and LV-miR-214-mi) were also purchased from GenePharma. HCT-116 cells were infected with the indicated lenti-virus particles of the same MOI = 5 for 24 hours and then stable over-expression cells were selected with the medium containing puromycin for at least two week.

### In vivo xenograft tumor growth

Five-week-old male BALB/cA-nu (nu/nu) nude mice were obtained from Nanjing Experimental Animal Center and maintained in pathogen-free conditions. The mice were separated into two groups and were subcutaneously injected with control (LV-NC-mi)-or miR-214 lenti-virus (LV-miR-214-mi)-infected HCT116 cells ( $5 \times 10^6$ ) at each flank. Tumor volumes were measured with a caliper every four days. When palpable tumors were formed for 40 days, the mice were killed and tumors were harvested and weighted.

### Statistic analysis

Data expressed as the mean  $\pm$  SEM were analyzed by SAS V8; paired t-test was used for comparison of clinical samples data and grouped t-test for other data.  $P < 0.05$  was considered statistically significant.

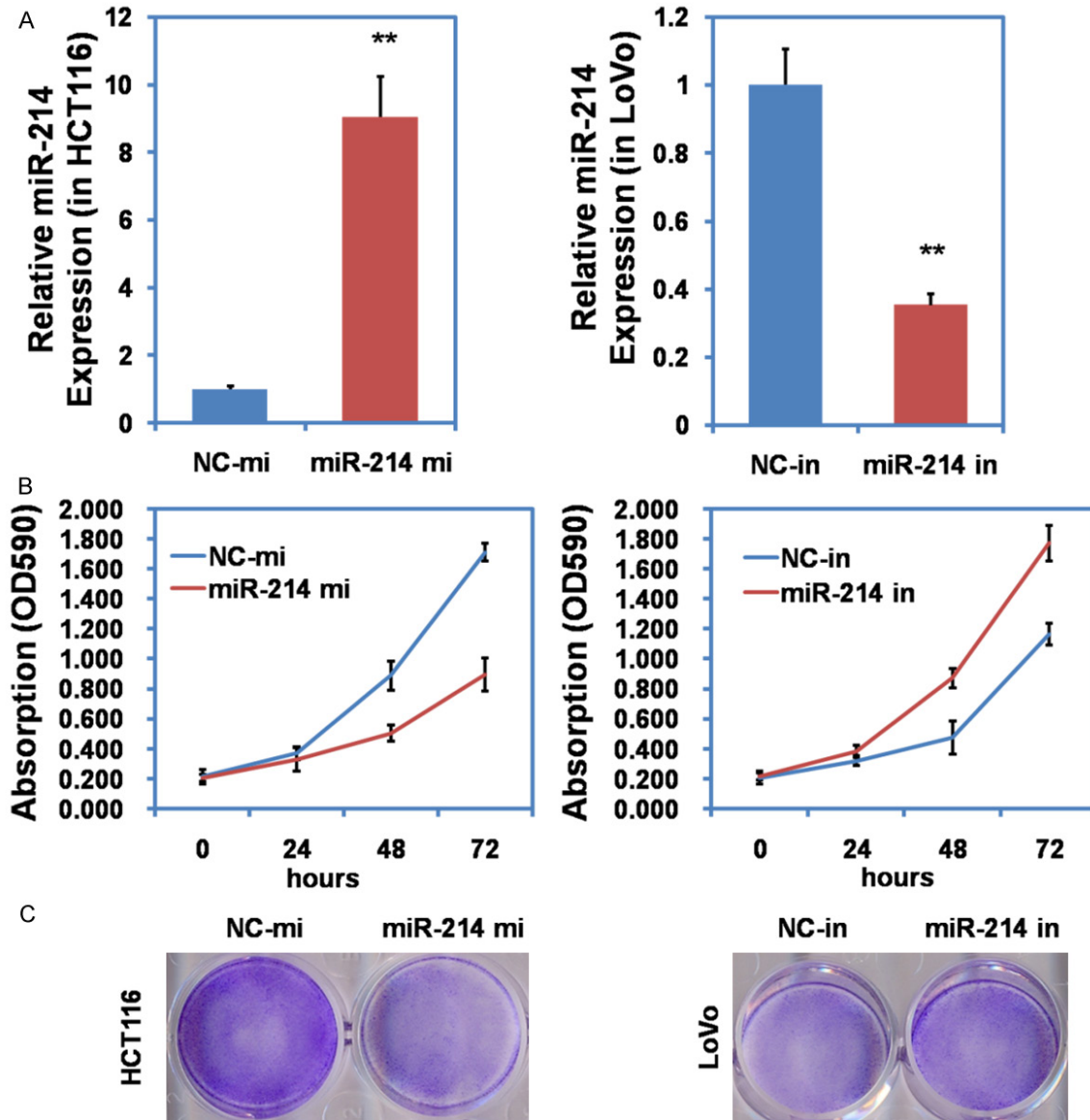
## Results

### MiR-214 is frequently attenuated in CC samples and cell lines

Although it was known that miR-214 was decreased in CC tissues [12, 13], we still started from examining its expression level in clinical CC samples. 25 pairs of CC frozen tissues and matched adjacent normal specimens were collected and then qPCR was performed to detect miR-214 expression in these paired normal and CC tissues. As shown in **Figure 1A**, we found that miR-214 was significantly under-expressed in CC tissues than that of the noncancerous specimens. Meanwhile, we also detected miR-214 expression in several CC cell lines (HCT-116, LS174T, Caco-2, SW480, SW620, LoVo and RKO). MiR-214 was also confirmed to be down-regulated in CC cell lines, when compared to non-cancerous fetal colon epithelial FHC cells (**Figure 1B**). These findings manifest that miR-214 may play a tumor-suppressive role in CC and its down-regulation be embroiled in cancer progression. Our results validated its expression pattern in CC.

### MiR-214 suppresses cell growth and viability in vitro

To investigate the functional role of miR-214 down-regulation in CC, CCK-8 assay was firstly done to evaluate the cell growth ability by miR-



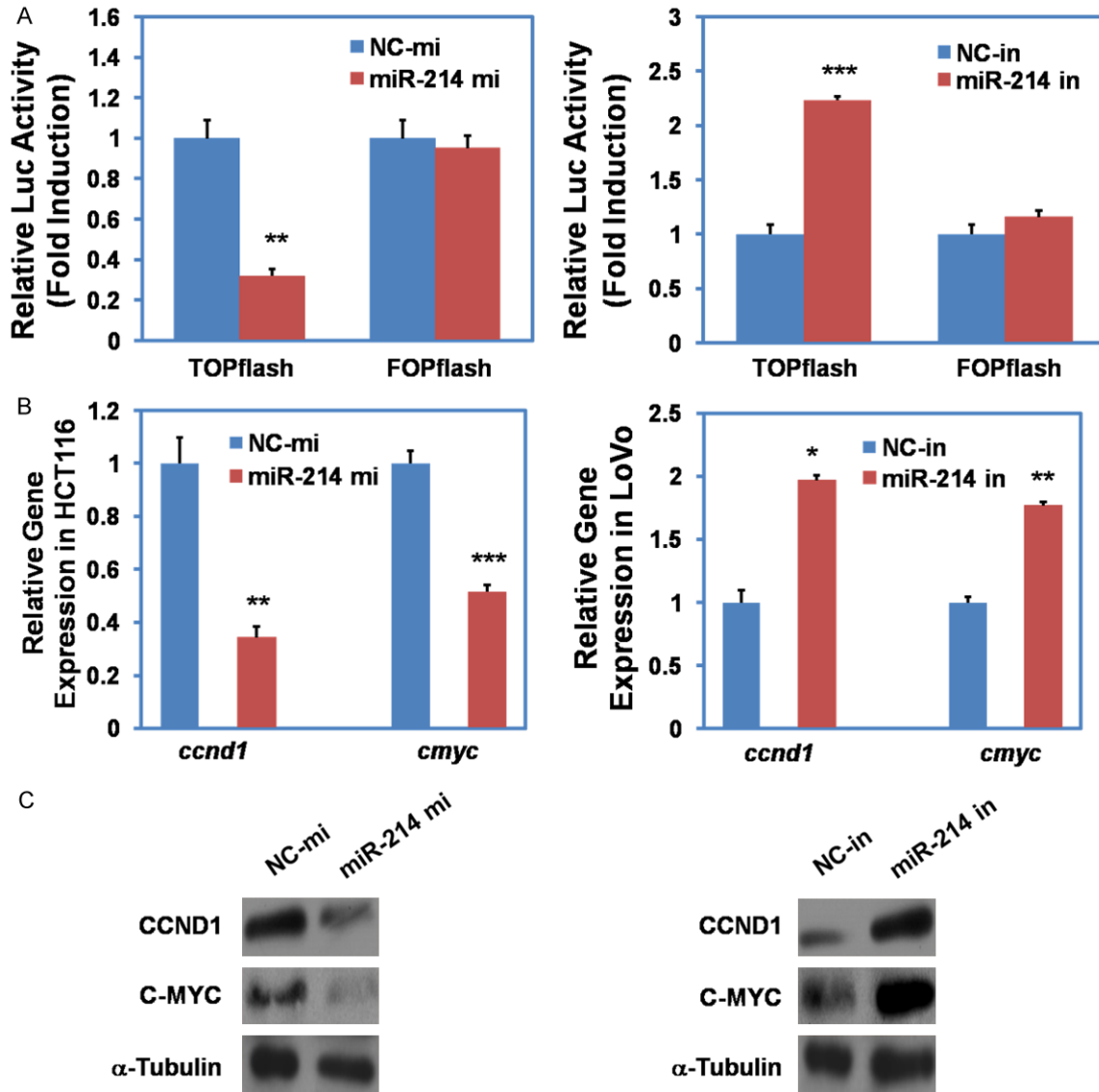
**Figure 2.** MiR-214 suppresses cell growth and viability in vitro. A. The over-expression or knock down effect of miR-214 in HCT116 or LoVo cells was assessed by qPCR. \*\*:  $P < 0.01$ , compared to control group. B. Inhibition or promoting of cell proliferation in HCT116 or LoVo cells after miR-214 over-expression or knockdown. Cell proliferation was measured using CCK-8 assay at 590 nm. Data had three repeats. C. After transfection of miR-214 mimics or inhibitors, HCT116 or LoVo cells were stained with crystal violet as described in Methods and Materials.

214 reintroduction/inhibition. The over-expression efficiency of miR-214 in the HCT116 and the knockdown efficiency in LoVo cells were achieved by respectively treated with miR-214 mimics and inhibitors (Figure 2A). The results from the CCK-8 assay showed that miR-214 reintroduction suppressed cell growth, while its inhibition accelerated cellular growth (Figure 2B). In addition, we also performed crystal staining 48 hrs after indicated transfection. As indicated in Figure 2C, significant cell viability inhibitions were observed in miR-214-overex-

pressing HCT116 cells compared with control. We also observed that miR-214-inhibited LoVo cells exhibited elevated cell viability. These observations demonstrate that miR-214 suppresses the oncogenicity of CC cells in vitro.

*MiR-214 exerts a negative effect on cell growth by regulating the Wnt/ $\beta$ -catenin pathway*

Next, in order to elucidate the mechanisms responsible for the suppressing effects of miR-



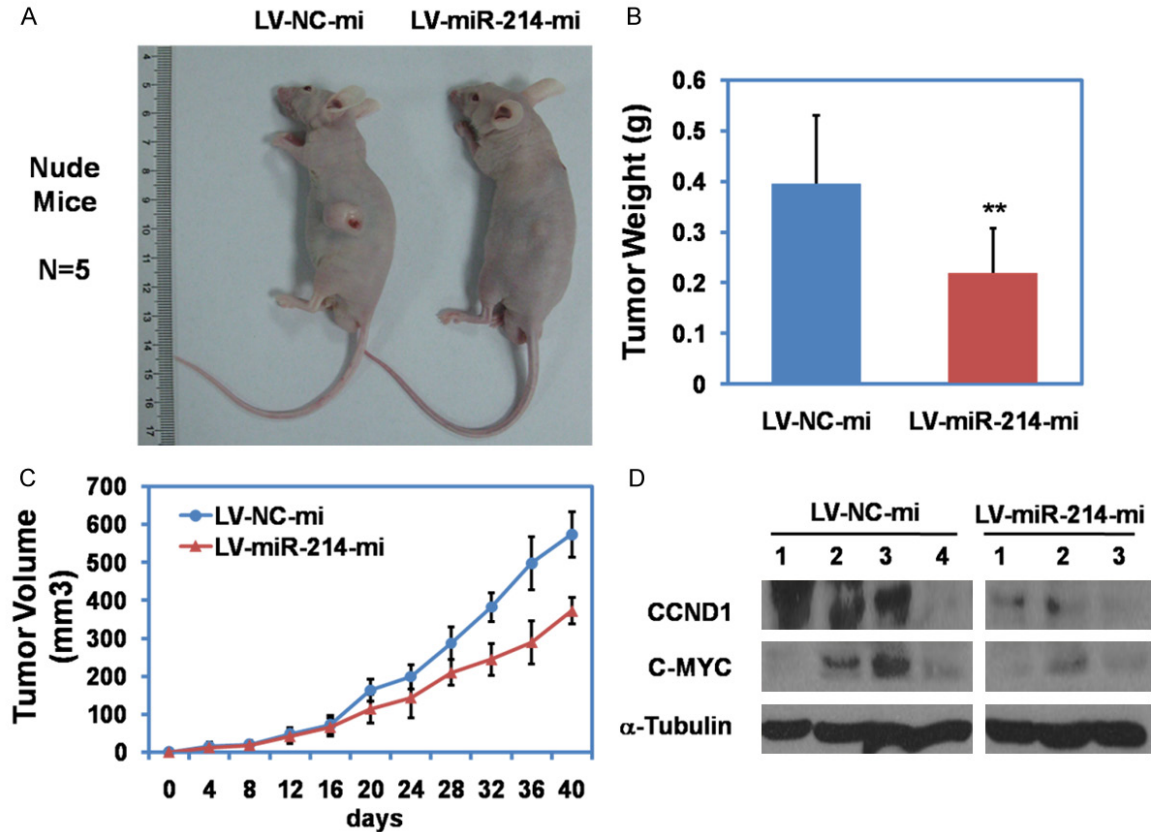
**Figure 3.** MiR-214 negatively regulated Wnt/ $\beta$ -catenin signaling pathway. **A.** Overexpression or knock down of miR-214 attenuated or enhanced Wnt/ $\beta$ -catenin signaling activity. HCT116 or LoVo cells were transfected with TOP flash (or FOP flash) luciferase reporter plasmid, renilla luciferase reporter plasmid, and miR-214 mimics or inhibitors. In 48 hours, luciferase activity was determined, and relative Wnt/ $\beta$ -catenin activity was calculated. \*\*:  $P < 0.01$ . \*\*\*:  $P < 0.001$ . **B, C.** MiR-214 mimics or inhibitors repressed or activated transcription of endogenous CCND1 and CMYC genes. HCT116 or LoVo cells were transfected with miR-214 mimics or inhibitors for 48 h, and then the cells were harvested for qPCR or western blot. \*:  $P < 0.05$ , \*\*:  $P < 0.01$ , \*\*\*:  $P < 0.001$ .

214 on cell growth, the activation of the Wnt/ $\beta$ -catenin pathway was examined. As shown in **Figure 3A**, miR-214 over-expression significantly reduced the TOP/FOP transcription activity. While inhibition of miR-214 led to elevated TOP flash activity. Moreover, the expression of  $\beta$ -catenin targeting cyclin D1, c-myc expression was determined by both qPCR and western blot. We observed that in miR-214-reintroduced cells, the expression of cyclin D1 and c-myc were obviously lower than those in

control cells, and vice versa (**Figure 3B** and **3C**). Thus, these findings prompted us to hypothesize that the Wnt/ $\beta$ -catenin signaling pathway is associated with the suppressing effects of miR-214 on the growth of CC cells.

*MiR-214 impairs tumor growth in vivo*

Finally, we accessed the role of miR-214 in CC *in vivo*. From the above findings, we already known that miR-214 inhibited cell growth and



**Figure 4.** MiR-214 impairs tumor growth in vivo. A. Images of xenograft-bearing nude mice taken after the animals were killed at the end of the 40<sup>th</sup> day. B. Tumors were harvested at the 40<sup>th</sup> day for analysis of the differences mass. C. Tumor growth was monitored over the whole 40 days. D. Tumor samples were subjected to western blot analysis for CCND1 and CMYC expression. Data are means  $\pm$  SEM, N = 5, \*\*:  $P < 0.01$ .

viability of CC cells. Then, tumor formation was done in a xerograph model by introducing miR-214-overexpressing HCT116 cells and control cells. Tumor weight and volume were recorded and expressions of related molecules were analyzed. We found that miR-214 significantly reduced tumor formation in mice (Figure 4A). Also, tumor weight and volume were both reduced in miR-214-overexpressing group (Figure 4B and 4C). Most importantly, we observed that in miR-214-stably-introduced tumors, our *in vitro* findings of Wnt/ $\beta$ -catenin signaling inhibition could also be reproduced *in vivo*, since down-regulation of cyclin D1 and c-myc were observed in miR-214-introduced tumors (Figure 4D). Above all, our data further indicated the tumor suppressing role of miR-214 in CC.

**Discussion**

Human CC is the third most commonly diagnosed malignant disease [14]. Recent develop-

ment in the therapeutic strategies has helped to cure many patients with early-stage of this disease. However, the prognosis of patients with advanced CC and metastasis is still poor, with 5-year survival rates for stage III and stage IV CC of 65.4 and 12.8%, respectively [15]. Therefore, improved understanding of the molecular basis of CC, and development of new strategies for better prevention and therapy of CC are attracting increased attention.

Aberrant activation of Wnt signaling, arising from genetic defects in the tumor suppressor genes APC or AXIN1/2, or activating mutations in  $\beta$ -catenin, has been associated with the initiation of numerous types of cancer, and is common in CC [16]. A primary consequence of Wnt signaling activation is the stabilization of  $\beta$ -catenin in the cytoplasm, resulting in increased translocation of  $\beta$ -catenin to the nucleus, thereby activating downstream target gene transcription. Published data showed that miR-214 was downregulated in CC and functio-

ned as a tumor suppressor [12, 13]. It remained unclear whether miR-214 regulated Wnt/ $\beta$ -catenin signaling in CC cells.

Until recently, only a few studies have linked miR-214 with the Wnt/ $\beta$ -catenin signaling in other types of cancers. Specifically, Xu et al. found that in esophageal cancer, miR-214 bound to 3'-UTR of  $\beta$ -catenin mRNA to inhibit its translation. They demonstrated a previously unappreciated role for miR-214 in suppression of  $\beta$ -catenin-mediated EC cell growth and invasion [17]. On the other hand, Wei et al. reported that miR-214 contributed to cell self-renewal by directly targeting catenin beta interacting protein 1 (CTNNBIP1), a member of the Wnt signaling pathway in cancer stem-like cells of lung adenocarcinoma [18]. In this study, we found that miR-214 negatively impacted on the Wnt/ $\beta$ -catenin signaling, thus inhibited cell growth and viability both in vitro and in vivo. Although we did not go on to dig out the direct targets of miR-214, we believed that there might be several targets that contributed to Wnt/ $\beta$ -catenin signaling. The combined effects showed the net inhibitory activities of this pathway. Nevertheless, further research on miR-214 functional mechanisms in CC is required.

To summarize, our results showed that miR-214 was decreased in CC, and exerted anti-tumor roles in cell growth and viability by suppressing Wnt/ $\beta$ -catenin signaling. Our data provided another clue for addressing the tumor suppressing function of miR-214 in CC, and miR-214 may be a potential therapeutic targets for CC.

### Acknowledgements

This work was supported by: National Natural Science Foundation of China. Grant No. U1304816.

### Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Qiang Yao, Department of Oncology, Tianjin Union Medicine Center, No190 Jieyuan Road, Hongqiao District, Tianjin 300073, China. E-mail: yaoqiang\_umc@yeah.net

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