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miR-612 suppresses the stemness of liver cancer via Wnt/β-catenin signaling

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ABSTRACT

Previous research showed that microRNA-612 (miR-612) has inhibitory effects on cell proliferation, migration, invasion, and metastasis of hepatocellular carcinoma (HCC). AKT2 was confirmed to be a direct target of miR-612, through which the epithelial–mesenchymal transition (EMT) and metastasis of HCC were inhibited. Our present findings reveal that miR-612 is able to suppress the stemness of HCC by reducing the number and size of tumorspheres as well as clone formation in soft agar, and to relieve drug resistance to cisplatin and 5-fluorouracil. In addition, miR-612 hampered the capacity of tumorigenesis in NOD/SCID mice and redistributed the tumor invasive frontier of miR-612-modulating cells. Finally, our findings suggest that Wnt/β-catenin signaling is required in the regulation of EMT-associated stem cell-like traits by miR-612.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third most common cause of cancer death globally [1]. Despite the improved understanding of tumor biology, the treatment efficacy in liver cancers has not improved greatly over the past decade. Cancer stem cells (CSCs) are defined as cells that possess the capacity to self-renew and differentiate into the heterogeneous lineages of cancer cells in a tumor [2]. The first conclusive evidence of the existence of CSCs was published in 1997 by Bonnet and Dick, who studied acute myelogenous leukemia [3]. More recently, CSCs have been isolated from many solid tumors, including HCC [4]. The existence of CSCs offers a reasonable explanation as to why current treatments targeting rapidly dividing cells fail to cure cancer patients. In fact, some evidence has directly attributed the therapeutic resistance to putative CSCs of solid tumors.

MicroRNAs (miRNAs) are endogenous noncoding RNAs that are post-transcriptional regulators of gene expression [5]. Emerging evidence suggests that several miRNAs may distinctively regulate the key biological properties of CSCs, including self-renewal and tumorigenesis [6]. In our previous study, miR-612 exhibited a pleiotropic inhibitory role on the HCC invasive-metastatic cascade, especially by suppressing cell proliferation, the epithelial–mesenchymal transition (EMT), local invasion, and distant colonization [7]. All these biological properties of miR-612 suggested to us that this miRNA could be a regulator of HCC stemness through an EMT-associated mechanism. Therefore, in this study we attempted to identify the possible roles of miR-612 in HCC stemness.

2. Materials and methods

2.1. Cell lines

HCCLM3 was established by our institute and kept in the cell bank of the Liver Cancer Institute, Zhongshan Hospital, Shanghai. HepG2 was purchased from the Shanghai Cell Bank, Chinese Academy of Sciences (CAS). HCCLM3 cells have a relatively high metastatic potential and express a low endogenous level of miR-612, whereas HepG2 cells have a lower metastatic potential and express

Abbreviations: miR-612, microRNA 612; 5-FU, 5-fluorouracil; CSCs, cancer stem cells; EMT, epithelial–mesenchymal transition; HCC, hepatocellular carcinoma.

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a higher endogenous level of miR-612. Both cell lines were cultured under standard conditions.

2.2. Oligonucleotides and transfection

Oligonucleotides including miRIDIAN miR-612 hairpin inhibitor, mimic, and negative control (Thermo Fisher Scientific) for miR-612 inhibition and restoration were used in this study. All these oligonucleotides were transfected into HCC cells at a final concentration of 100 nM using Lipofectamine 2000 according to the product manual (Invitrogen) [7].

2.3. Stable over- and knocked-down expression of miR-612 with lentiviral vectors

The human pre-miR-612 gene was cloned into hU6-MCS-CMV-EGFP for ectopic overexpression of miR-612 (miR-612-o) and hU6-MCS-CMV-EGFP as its corresponding negative control (miR-612-o-mock). The reverse complement sequence of miR-612 was cloned into hU6-MCS-CMV-RFP for knocked-down expression of miR-612 (miR-612-i) and hU6-MCS-CMV-RFP as its corresponding negative control (miR-612-i-mock). HCCLM3 and HepG2 cells were infected with above lentiviral constructs, respectively, using Lipofectamine 2000. The transfection efficiency was confirmed by fluorescence intensities of GFP and RFP, as well as the levels of miR-612 and AKT2.

2.4. RNA extraction and real-time PCR assays for miR-612, cyclin D1, and c-Myc detection

Real-time PCR was conducted essentially as described [7]. Real-time PCR was performed by the SYBR Green PCR method using an All-in-One miRNA qPCR Detection Kit (GeneCopoeia, Inc.) for miRNA detection and SYBR Premix Ex Taq for mRNA detection (TaKaRa, Inc.).

2.5. Protein levels detected by Western blot analysis

Western blot was conducted as described [7] using anti-AKT2 (1:4000, Abcam), β-catenin (1:5000, Abcam), cyclin D1 (1:1000, Proteintech), c-Myc (1:500, Proteintech) antibodies.

2.6. Tumorsphere assay

Tumorsphere assay was performed as described [8]. Only tumorspheres with a diameter larger than 50 μm were counted.

2.7. Clone formation assay

Experiment was performed as described [9]. Cell colonies l were photographed after 7 days of culture. Those with a diameter larger than 50 μm were counted.

2.8. In vitro cell cytotoxicity assays

Cell cytotoxicity was determined by the Cell Counting Kit-8 (Dojindo Laboratories) assay. In brief, miR-612-o HCCLM3 cells and miR-612-i HepG2 cells, as well as their corresponding mock cells, were separately seeded into 96-wells plates at an initial number of 5000 cells per well. After 24 h, the cells were treated with 0.05, 0.5, 5, 50, or 500 μg/ml of cisplatin or with 1, 10, 100, 1000, or 10000 μg/ml of 5-fluorouracil (5-FU) for 48 h. Ten microliters of the kit reagent was added to each well, and 2 h later all plates were scanned by a microplate reader (Thermo Fisher Scientific) at 450 nm. Cell cytotoxicity was calculated on the basis of absorbency.

2.9. Tumorigenicity in NOD/SCID mice and athymic nude mice

Four-week-old male NOD/SCID mice were purchased from Shanghai Institutes for Biological Sciences, CAS. Four-week-old male athymic BALB/C nude mice were purchased from Shanghai Laboratory Animal Company. For the tumorigenic assay, 2×10^5 (or 2×10^6) HCCLM3 and 2×10^4 (or 2×10^5) HepG2 cells were prepared in 200 μl of normal saline after the indicated treatment and subcutaneously injected into NOD/SCID mice. For the cell distribution assay, a cell mixture (2×10^6 cells) with equal numbers of miR-612-o HCC cells labeled with green fluorescent protein (GFP) and miR-612-i HCC cells labeled with red fluorescent protein (RFP) were injected into nude mice subcutaneously (miR-612-o+miR-612-i). As a control, the same numbers of miR-612-o-mock HCC cells (with GFP) and miR-612-i-mock HCC cells (with RFP) were injected in the same way (miR-612-o-mock+miR-612-i-mock). At the endpoint, all mice were sacrificed. Tumor sizes were calculated as volume (mm^3) = [width^2 (mm^2) × length (mm)]/2. Xenograft tumors from nude mice models were frozen and fixed in 100% acetone solution. All tissue sections, particularly those at the edge of tumors, were photographed using fluorescence microscopy. The protocol was approved by the Animal Care and Use Committee of Shanghai, China.

2.10. Indirect immunofluorescent assay

Immunofluorescence was conducted essentially as described [10] using an anti-β-catenin (Abcam) antibody at a dilution of 1:250.

2.11. Luciferase reporter assays

TOPflash reporter construct and Renilla luciferase construct were kindly provided by Prof. Li-Jian Hui (Shanghai Institutes for Biological Sciences, CAS). Luciferase activities of indicated cells were measured using the Dual-Luciferase Reporter Assay System (Promega). The levels of firefly luciferase activities were obtained by normalizing to Renilla luciferase activities and relative to a control, as previously reported [10].

2.12. Statistical analysis

Data were analyzed using GraphPad Prism 5 software [10]. Quantitative variables were expressed as means ± SD and analyzed by one-way ANOVA and Student's *t*-test. Results were considered statistically significant at $P < 0.05$.

3. Results

3.1. miR-612 suppresses tumorsphere and clone formation of HCC cells

Sphere-forming capability is a major property of normal stem cells as well as putative CSCs, and nonadherent tumorsphere assays are widely used to evaluate the self-renewal ability of CSCs. Therefore, we first evaluated tumorsphere formation of HCCLM3 and HepG2 cells after a 48-h treatment with miR-612 mimic (miR-612-o), miR-612 inhibitor (miR-612-i), and mock oligonucleotides (mock), respectively. We found that the number of tumorsphere (with a diameter larger than 50 μm) in miR-612-o-treated HCCLM3 cells were significantly less than those of mock and WT groups (0 ± 0 vs. 6.67 ± 1.15 and 5.33 ± 1.15; Fig. 1A and B), whereas the number and size of tumorspheres in miR-612-i-treated HepG2 cells were remarkably greater than those in mock and WT groups (14 ± 2 vs. 5.33 ± 1.15 and 4.67 ± 1.15; $P = 0.0078$; Fig. 1A and B). To confirm these results, the anchorage-

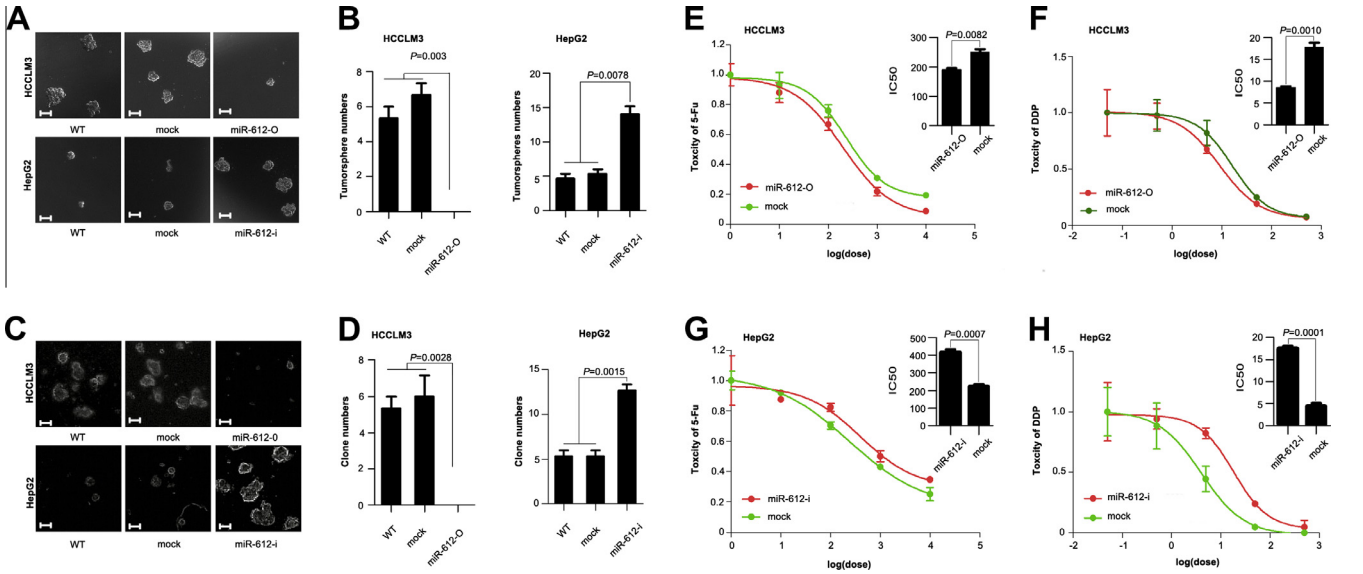

Fig. 1. miR-612 suppresses the stemness of HCC in vitro. (A, B) Representative images and statistical results of HCCLM3 and HepG2 tumorspheres (>50 μm) after indicated treatments ($n = 3$; magnification: 25×; scale bar: 50 μm). (C, D) Representative images and statistical results of HCCLM3 and HepG2 cell clones (>50 μm) with indicated treatments ($n = 3$; magnification: 5×. scale bar: 50 μm). (E, F) The nonlinear regression toxicity curves of HCCLM3 after treatment with cisplatin (DDP) and 5-FU. miR-612-o HCCLM3 was much more sensitive to DDP and 5-FU compared with its mock control ($n = 3$). (G, H) The nonlinear regression toxicity curves of HepG2 after treatment with DDP and 5-FU. miR-612-i HepG2 exhibited much more resistance to DDP and 5-FU compared with its mock control ($n = 3$).

independent growth assay in soft agar was used. Again, the clone formation ability of HCCLM3 cells was significantly inhibited by miR-612 mimic (miR-612-o) and the clone formation ability of HepG2 cells was obviously promoted by miR-612 inhibitor (miR-612-i) (Fig. 1C and D). Together these data suggest that miR-612 has a negative regulatory role on self-renewal of HCC.

3.2. miR-612 reduces drug resistance of HCC cells

Recent studies have revealed that chemoresistance is another major property of CSCs [11]. To investigate whether miR-612 has a regulatory role in the chemosensitivity on HCC cells, two chemotherapeutic drugs, 5-Fu and cisplatin, were used in this study. The IC50 values of 5-Fu and cisplatin in miR-612-m-treated HCCLM3 cells were 8.55 ± 0.52 and 190.83 ± 7.57 g/ml, respectively, which were significantly lower than that in control groups (mock; 249.57 ± 19.46 and 17.75 ± 1.76 g/ml; $P = 0.0010$ and $P = 0.0082$, respectively; Fig. 1E and F). In addition, the IC50 values of 5-Fu and cisplatin in miR-612-i-treated HepG2 cells were much higher than those of negative counterparts (mock; 416.53 ± 29.43 μg/ml vs. 223.40 ± 20.28 μg/ml for 5-FU and 17.52 ± 0.94 μg/ml vs. 4.45 ± 1.18 μg/ml for cisplatin; $P = 0.0007$ and $P = 0.0001$, respectively; Fig. 1G and H). The data showed that miR-612 can increase chemosensitivity of HCC cells to 5-Fu and cisplatin, suggesting that miR-612 negatively regulates HCC stemness.

3.3. miR-612 suppresses tumorigenesis of HCC in NOD/SCID mice

Because the tumorigenesis in vivo is the key parameter of CSCs, we next employed a NOD/SCID mice model and miR-612 inhibited or overexpressed HCC cells established previously to further evaluate the role of miR-612 on CSC formation in HCC [7]. In brief, we subcutaneously injected a small number of HCC cells with miR-612 premodification into NOD/SCID mice. Four weeks later, all mice (5/5 for each group) succeeded to grow xenograft tumors when an injection of 2×10^6 HCCLM3 cells, but the tumor size in the miR-612-o group was significantly smaller than that of the mock group (2425.58 ± 543.33 mm^3 vs. 3529.89 ± 369.92 mm^3; $P = 0.0056$; Fig. 2A and B). However, when we decreased the number of injected cells to 2×10^5, no xenograft tumor growth was found in the miR-612-o group, whereas xenograft tumors did develop in all mice (5/5) in the mock group (Fig. 2A and B). We then duplicated the above experiments with HepG2 cells, which have a low tumorigenesis rate and high level of miR-612. We found that upon injection of 2×10^4 HepG2 cells, the incidence rate of xenograft tumor in mice was 100% (5/5) in the miR-612-i group, while all mice failed to form xenograft tumors in the mock group (Fig. 2C and D). Again, all mice (5/5 for each group) succeeded to grow xenograft tumors after an injection of 2×10^5 HepG2 cells, but the tumor volume in the miR-612-i group was significantly larger than that in the mock group (2957.74 ± 984.50 mm^3 vs. 880.82 ± 320.47 mm^3; $P = 0.0020$; $n = 5$; Fig. 2C and D). These results indicating that miR-612 suppresses HCC tumorigenesis suggest that miR-612 can suppress HCC stemness.

3.4. miR-612 inhibits an invasive frontier of HCC xenografts

CSCs are not only associated with tumorigenesis, but they also initiate the tumor metastasis cascade. Tumor metastasis often occurs at the margins of tumors. In a previous study, we showed that the lower the level of miR-612, the more EMT HCC was located at the tumor margin, thus facilitating more local invasion [7]. In the present study, we employed a two-fluorescent xenograft model to provide direct evidence that miR-612 could inhibit the formation of invasive protrusion. Equal numbers of miR-612-o and miR-612-i HCCLM3 cells marked with GFP and RFP, respectively, were mixed and then injected subcutaneously into nude mice, as well as their counterpart negative control (mock). Four weeks later, a disequilibrium distribution of miR-612-o and miR-612-i cells was easily observed at the frontier of HCCLM3 xenograft tumor sections, where miR-612-i HCCLM3 cells were dominant (Fig. 2D, indicated by an arrow). However, a nearly equilibrium distribution of miR-612-o-mock and miR-612-i-mock cells at the edges of HCCLM3 xenografts was observed (Fig. 2D). The imbalance of cell distribution on the margin of tumor was also confirmed in HepG2 xenografts (Fig. 2D), indicating that miR-612 directly suppresses

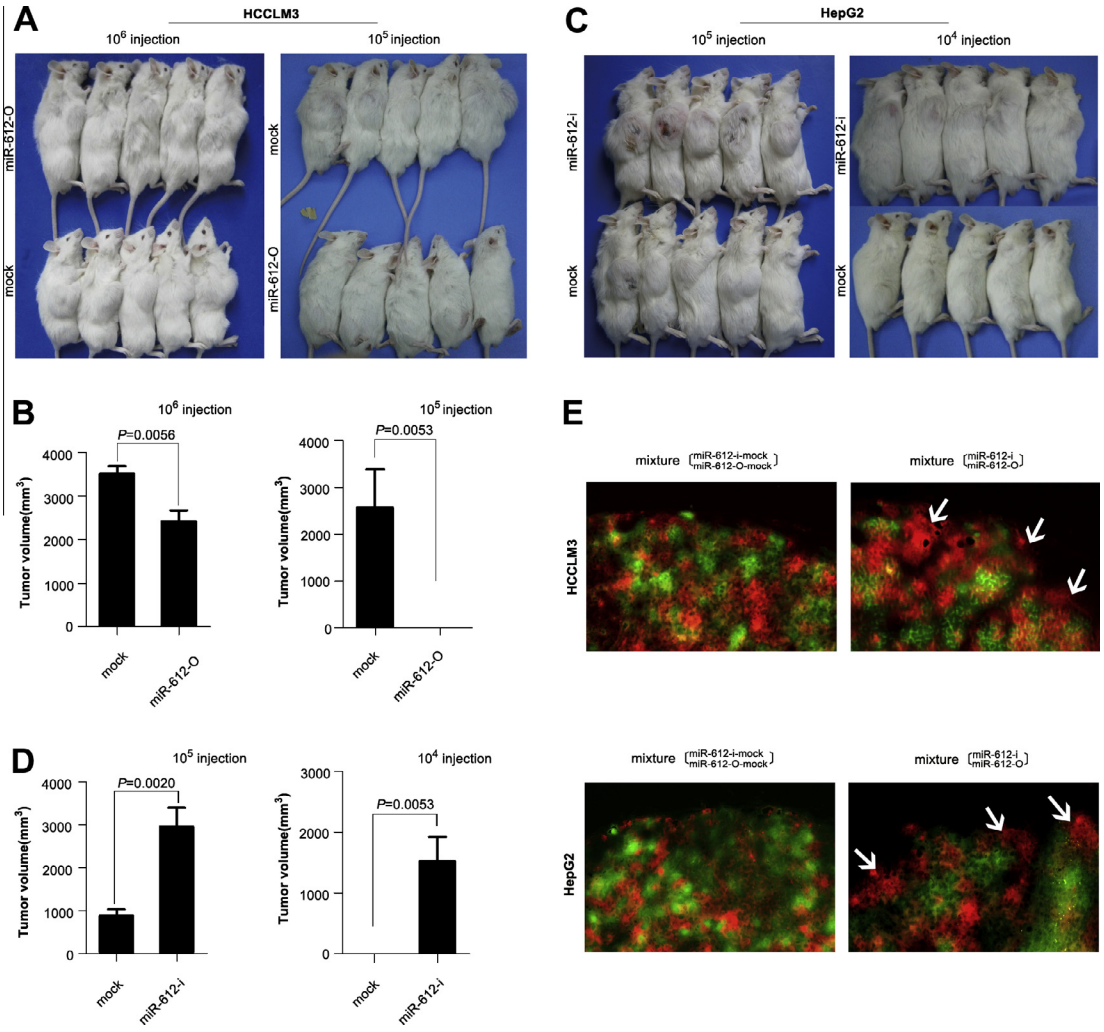

Fig. 2. miR-612 modulates tumorigenicity and the invasive frontier of HCC in vivo. (A, B) miR-612-o HCCLM3 cells (2×10^6) initiated smaller subcutaneous tumors than those of mock counterparts (A, left; $n = 5$). All NOD/SCID mice (5/5) were successfully grafted using 2×10^5 mock cells, but failed using miR-612-O HCCLM3 cells (A, right, $n = 5$). (C, D) miR-612-i HepG2 cells (2×10^5) initiated larger subcutaneous tumors than those of mock counterparts (C, left, $n = 5$). All NOD/SCID mice (5/5) were successfully grafted using 2×10^4 miR-612-i HepG2 cells, whereas grafting failed using mock cells (C, right; $n = 5$). (E) A disequilibrium distribution of miR-612-o and miR-612-i cells was observed at tumor edges. The invasive protrusion was mainly composed of miR-612-i HCCLM3 and HepG2 cells (right, arrow). An equilibrium distribution of miR-612-o-mock and miR-612-i-mock cells was found at the tumor frontier (left).

invasive protrusion formation, and hence decreases both local and distant metastasis of HCC.

3.5. miR-612 suppresses Wnt/β-catenin signaling

Wnt signaling plays a pivotal role in maintaining the biological properties of progenitors and stem cells. Dysregulation of Wnt signaling promotes the initiation and progression of various human cancers. Based on our previous results, miR-612 modulates EMT of HCC by directly targeting AKT2 accompanied by up-regulation of E-cadherin [7]. Therefore, we hypothesized that miR-612 may regulate Wnt signaling and the stemness of HCC as well.

In this study, the total protein levels of β-catenin in HCCLM3 and HepG2 cells were analyzed by Western blot assays, but they exhibited no obvious changes in the cells after up- and down-regulation of miR-612 (Fig. 3A and B). Because E-cadherin can sequester a large amount of β-catenin at the inner cytoplasmic membrane [12], we wondered whether the decline of E-cadherin induced by miR-612 would release β-catenin and promote its nuclear translocation. Therefore, the subcellular localization of β-catenin was assayed by an immunofluorescent method. Indeed, miR-612 did affect the subcellular localization of β-catenin. HCCLM3 cells treated with miR-612-o exhibited much more cytoplasmic localization of β-catenin than mock cells (Fig. 3C). On the contrary, HepG2 cells treated with miR-612-i resulted in much more nuclear localization of β-catenin than the mock control (Fig. 3C).

To further observe the biological functions of β-catenin redistribution, we performed a reporter assay using TOPflash and Renilla constructs with multiple TCF/LEF-binding sites in the promoter of firefly luciferase reporter gene. The luciferase activities in HCCLM3 cells with miR-612-o treatment were 4-fold lower than that in the mock group ($P = 0.0009$, Fig. 3D), whereas the luciferase activities in miR-612-i-treated HepG2 cells markedly increased compared with the control group ($P = 0.00187$, Fig. 3D).

In addition, cyclin D1 and c-Myc, two endogenous targets of Wnt signaling, were negatively regulated by miR-612. The mRNA and protein levels of cyclin D1 were significantly down-regulated after miR-612-o treatment in HCCLM3 cells and up-regulated after miR-612-i treatment in HepG2 cells (Fig. 3E and F). These data indicate that miR-612 played a suppressive role on the transcriptional activities of TCF/LEF and then Wnt/β-catenin signaling in HCC.

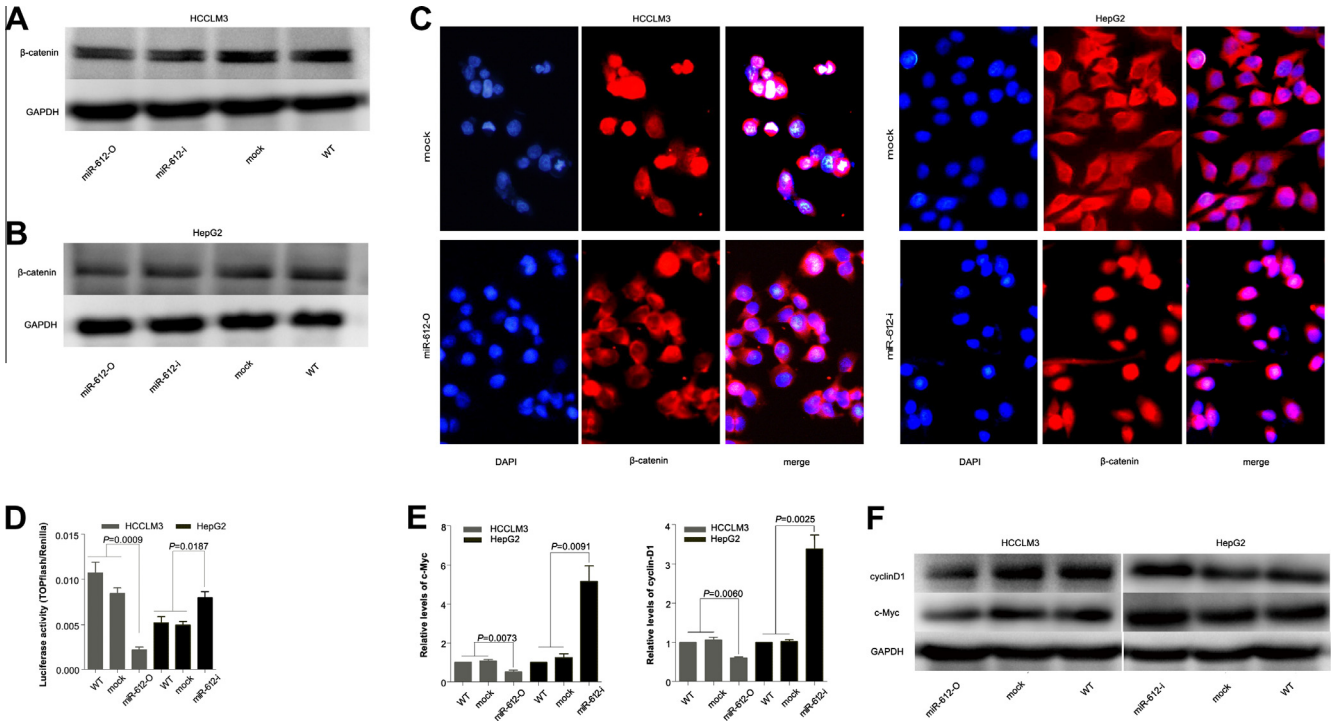

Fig. 3. Wnt/β-catenin signaling regulates the stemness of HCC cells. (A, B) The protein levels of β-catenin in HCCLM3 and HepG2 cells after indicated treatments exhibited no obvious changes. (C) β-Catenin in miR-612-o HCCLM3 cells exhibited much more cytoplasmic localization, whereas β-catenin in miR-612-i HepG2 cells exhibited much more nuclear localization than their mock counterparts. (D) Decreasing and increasing activities of TCF/LEF were observed in miR-612-o HCCLM3 and miR-612-i HepG2 cells, respectively ($n = 3$). (E) Down- and up-regulated mRNA levels of endogenous cyclin D1 and c-Myc were found in miR-612-o HCCLM3 and miR-612-i HepG2 cells, respectively ($n = 3$). (F) Significantly down- and up-regulated protein levels of cyclin D1 and c-Myc were found in miR-612-o HCCLM3 and miR-612-i HepG2 cells, respectively ($n = 3$).

Finally, tumorsphere assays were performed to further identify the roles of miR-612 in tumor cell self-renewal through Wnt signaling. The average numbers of HCCLM3 tumorspheres pretreated with Wnt3α (2 μg/ml), BIO (2 μM), miR-612-o, miR-612-o+Wnt3α, miR-612-o+BIO and WT were 11.33 ± 1.15, 10 ± 2, 0 ± 0, 4 ± 2, 5.33 ± 1.15, and 4.3 ± 1.5, respectively. We found significant differences in tumorsphere numbers between the Wnt3α and miR-612-o+Wnt3α groups ($P = 0.0053$) and the BIO and miR-612-o+BIO groups ($P = 0.0249$; Fig. 4A). Also, the sizes of tumorspheres in the Wnt3α and BIO groups were larger than those in the miR-612-o+Wnt3α and miR-612-o+BIO groups (Fig. 4B). Therefore, miR-612 indeed reversed the promoting effects of Wnt3α and BIO on HCC tumorigenesis. The results also indicated that miR-612 did suppress HCC self-renewal via Wnt signaling.

4. Discussion

MicroRNAs are good molecular biomarkers for cancer diagnosis, prognosis, and therapy, and function as regulators of many oncobiological processes, such as CSC division and differentiation, tumorigenesis, EMT, and tumor metastasis [13]. The stemness of cancer cells has been proven to be regulated by the miRNA pathway [14].

The suppressive effects of miR-612 on HCC proliferation, EMT, metastatic colonization, and growth suggested that miR-612 could be a key regulator of the stemness of HCC. To confirm this hypothesis, we planned to investigate the role of miR-612 on HCC stemness, we used four functional assays to test the roles of miR-612 on HCC stemness in this study. Indeed, we identified for the first time that miR-612 plays a suppressive role on tumorsphere and

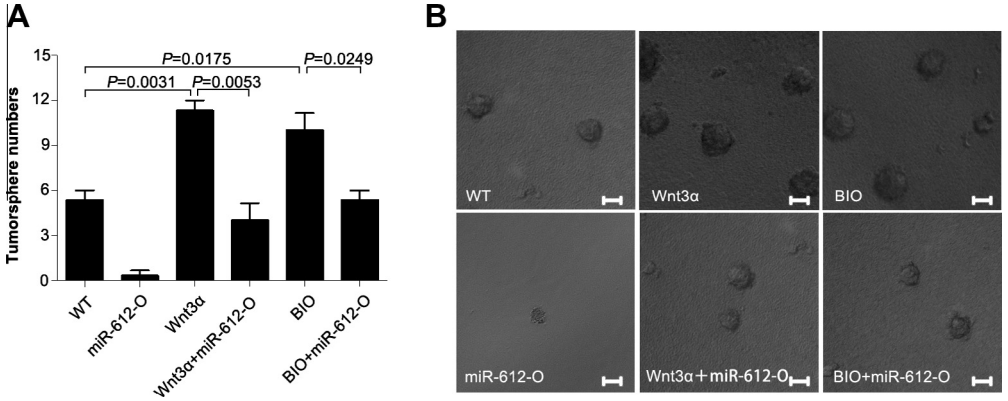

Fig. 4. miR-612 reverse the promoting effects of Wnt3α and BIO on tumorsphere formation via Wnt signaling. (A) The statistical results of tumorspheres (>50 μm) were calculated based on three independent experiments. The promoting effects of Wnt3α and BIO on tumorsphere formation are reversed by overexpression of miR-612. (B) Representative images of tumorspheres (>50 μm) of HCCLM3 cells pretreated with none (WT), Wnt3α (2 μg/ml), BIO (2 μM), miR-612-o, miR-612-o+Wnt3α, or miR-612-o+BIO (magnification: 50×; scale bar: 50 μm).

clone formation of HCCLM3 and HepG2 cells within in vitro systems. We also showed that miR-612 partly reversed HCC resistance to cisplatin and 5-FU. In addition, miR-612 was able to suppress tumorigenicity of HCCLM3 and HepG2 cells in NOD/SCID mice, especially when the tumor cells were injected subcutaneously at a low number of 2×10^4 cells. These in vitro and in vivo results together indicated that miR-612 was able to regulate the stemness of HCC.

As accumulating evidence supports that CSCs are the source of aggressive features in many solid tumors [15], liver CSCs have been found not only to play a major role in initiating and sustaining HCC primary tumors but also in facilitating HCC metastasis to distant organs [16,17]. In addition, a trace of tumor cells with CSC biomarkers has been found at the edge of pancreatic tumor, which facilitated human pancreatic tumor metastases [18]. Therefore, we further investigated the role of miR-612 on the formation of tumor invasive frontier using two fluorescent HCC xenograft models. When a cell mixture with equal numbers of miR-612-o and miR-612-i HCCLM3 or HepG2 cells was injected subcutaneously into nude mice, more miR-612-i HCCLM3 and HepG2 cells were present at the invasive protrusion of the tumor frontier. Together with our previous observations that miR-612 negatively modulated the local invasion in orthotopic HCCLM3 xenograft, this finding provides more direct evidence that miR-612 inhibits HCC invasion, the first step of tumor metastasis, by forming an asymmetric invasive protrusion mainly with miR-612-i HCC cells.

Recent studies revealed the cross-talk between β-catenin signaling and other pathways, such as PI3K/AKT pathway, a major pathway of EMT signaling, in various cancers. Because AKT2 was verified as a direct target of miR-612 in our previous study [7], we focused our concerns on Wnt signaling for the two reasons: Wnt/β-catenin signaling plays a crucial role in CSC regulation, and it is theoretically possible that AKT2 regulates β-catenin signaling though the AKT2/GSK-3β/β-catenin pathway and thus may impact the stemness of HCC. After treatments with miR-612 mimic or inhibitor, the total protein level of β-catenin in HCCLM3 and HepG2 cells was not different than that of mock and untreated cells. This result indicates that β-catenin could not be a direct target of miR-612.

Next we explored the indirect role of miR-612 on Wnt signaling. Because a large amount of β-catenin can be sequestered at the inner cytoplasmic membrane by E-cadherin, any changes in the E-cadherin expression level in HCC cells will theoretically affect the subcellular localization and nuclear translocation of β-catenin, and thus its transcriptional activity of the TCF/LEF complex. During the EMT of HCC, E-cadherin was found to be negatively regulated by miR-612. For example, a higher level of E-cadherin was found in miR-612-overexpressed HCCLM3 cells, whereas a lower level of E-cadherin existed in miR-612 knocked-down HepG2 and SMMC-7721 cells compared with their control cells. Therefore, an immunofluorescent assay was used in this study to monitor the subcellular localization of β-catenin in miR-612-o-treated HCCLM3 cells and miR-612-i-treated HepG2 cells. As expected, overexpression of miR-612 indeed sequestered a large amount of β-catenin at the inner cytoplasmic membrane of HCCLM3 cells, whereas downregulation of miR-612 promoted nuclear translocation of β-catenin in HepG2 cells. Furthermore, the transcriptional activity of TCF/LEF was negatively regulated by miR-612, according to a TOPflash reporter assay. Of more importance, two putative endogenous target genes of Wnt signaling, cyclin D1 and c-Myc, were found to be negatively modulated after miR-612 treatments in HCCLM3 and HepG2 cells. In addition, the stimuli of Wnt3α and BIO on tumorsphere formation could be partly reversed by miR-612. All these findings indicate that Wnt/β-catenin signaling is involved in the regulation of HCC stemness by miR-612.

In conclusion, we provide more evidence that miR-612 negatively regulates CSCs of HCC through Wnt/β-catenin signaling. The pleiotropic roles of miR-612 on HCC EMT and CSCs suggest that it could be an effective molecular target for HCC therapy.

Acknowledgments

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