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c-MYC-Regulated miRNA-92b Inhibits Expression of The E3 Ligase FBXW7 in Colorectal Cancer*

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Abbreviations: CRC, colorectal cancer; FBXW7, F-Box and WD repeat domain-containing 7; NLK, Nemo-like kinase; NSCLC, non-small cell lung cancer; RECK, reversion-inducing cysteine-rich protein with kazal motifs; CMV, cytomegalovirus; DKK3, Dickkopf-3; PTEN, phosphatase and tensin homolog deleted on chromosome ten.

Abstract Colorectal cancer (CRC) is the third leading cause of cancer-related death worldwide. Many microRNAs (miRNAs) have been reported to be abnormally expressed in CRC. Recent studies have identified miR-92b as a potential oncogene in several types of cancer. However, the role of miR-92b in CRC has not been clarified. This study aimed to elucidate the role of miR-92b in CRC progression. Relative quantitative PCR revealed that miR-92b expression was significantly increased in human CRC tissues compared to the adjacent tissues. Overexpression of miR-92b in the colorectal cancer cell line SW620 substantially increased cell viability *in vitro* and xenograft tumor growth *in vivo*. Also, miR-92b was identified as a secreted miRNA, which can be detected in both cultured medium and the peripheral blood of xenograft mice. Furthermore, we demonstrated that c-MYC, which was also elevated in CRC tissues, promoted the transcription of miR-92b by regulating its promoter activity. Luciferase assay and Western blot analysis revealed that FBXW7 was a novel target of miR-92b and can be negatively regulated by c-MYC. As FBXW7 is a major E3 ligase of c-MYC, our data suggested a potential positive regulatory feedback loop among c-MYC, miR-92b and FBXW7 in CRC. Collectively, we partly provided evidence on how miR-92b is regulated and the potential implications of miR-92b in CRC diagnosis.

Key words miR-92b, colorectal cancer, secretory miRNA, c-MYC, FBXW7
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Colorectal cancer (CRC) is the third most common cancer in the world, with approximately 1.4 million new cases diagnosed in 2012, which represent nearly 10% of the global cancer burden, and 694 000 deaths from the disease were reported (WHO International Agency for Research on Cancer. GLOBOCAN 2012). The incidence and mortality of CRC are also increasing in China [1–2]. In general, colorectal cancer is a highly curable disease if diagnosed at an early stage. Identifying more sensitive, unique and easy-to-test biomarkers for CRC diagnosis and prognosis prediction is urgently needed[3].

MicroRNAs (miRNAs) are a class of small non-coding RNAs of 18～24 nucleotides in length that

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regulate the translation and stability of specific target mRNAs[4–5]. The ectopic expression of miRNAs, along with their profiles in human cancers, could be used to classify tumors and improve prediction, prognosis and progression[6–7]. Recently, a growing body of evidence has demonstrated that miRNAs are consistently and reproducibly altered in CRC[8]. For example, precancerous and neoplastic colorectal tissues exhibit reduced levels of miR-143 and miR-145 compared to normal tissue, suggesting that these miRNAs may be tumor suppressors[9]. Another CRC-associated miRNA, miR-21, is a potential oncogenic miRNA expressed at high levels in tumor tissues[10]. Many other miRNAs have also been found to be deregulated in CRC, including miR-203[11] and miR-135a/b[12].

Up-regulation of miR-92b is observed in several cancers and contributes to cancer progression. Primary brain tumors express higher levels of miR-92b compared to primary breast, kidney and ovary tumors and their corresponding brain metastases[13]. In glioma, miR-92b was reported to control proliferation and invasion through activation of Wnt/beta-catenin signaling by targeting Nemo-like kinase (NLK)[14]. In addition, inhibition of miR-92b strongly suppressed cell proliferation, migration, and invasion of non-small cell lung cancer (NSCLC) cells, and expression of miR-92b was negatively correlated with its target, reversion-inducing cysteine-rich protein with kazal motifs (RECK), in NSCLC tissues[15]. Considering the important role of miR-92b in various cancers, we chose to investigate the potential role of miR-92b in colorectal cancer progression.

The proto-oncogene c-MYC is a multifunctional protein that plays a role in growth control, cell cycle progression and cellular transformation and is overexpressed in a variety of human tumors, including lymphomas and cancers of mesenchymal and epithelial origin[16]. It functions as a transcription factor that regulates specific target genes involved in several cellular processes[17]. c-MYC protein is degraded by FBXW7 (F-Box and WD repeat domain-containing 7), a SCF ubiquitin ligase[18]. Accumulating data have indicated that FBXW7 functions as a tumor suppressor and is frequently mutated in human cancers[19]. In addition, it was reported that the FBXW7 mRNA level is significantly lower in tumor tissues than the corresponding normal tissues[20].

Here, we show that miR-92b is up-regulated in CRC compared to the adjacent tissues. The increased miR-92b promoted CRC cell viability *in vitro* and xenograft tumor growth *in vivo*. Additionally, we found that miR-92b secretion was increased by CRC cells both in cultured medium and the peripheral blood of xenograft mice. Furthermore, we demonstrated that c-MYC could directly promote the transcription of miR-92b. miR-92b, in turn, regulated protein levels through direct binding to the 3′ UTR of the FBXW7 mRNA. Together, our results suggest that c-MYC-regulated miR-92b up-regulation promoted CRC tumorigenesis and disease progression and identified miR-92b as a potential therapeutic target and a CRC diagnostic biomarker.

1 Materials and methods

1.1 Tissue sample

Pairs of primary colorectal tumor tissues and adjacent non-tumor tissue samples from 20 patients were provided by the Tumor Biobank of the Cancer Institute, Zhejiang University. The samples were obtained by surgical resection and stored at –80℃ at the Second Affiliated Hospital of Zhejiang University School of Medicine. Consent forms were signed by all patients. The procedures were approved by the Ethical Committee of the Second Affiliated Hospital of Zhejiang University, School of Medicine. Both normal and tumor tissues were verified by histological analysis.

1.2 Cell culture and reagents

HEK 293T cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Biochrom, Berlin, Germany). The human CRC cell line SW620 was cultured in Leibovitz's L-15 medium (Invitrogen) supplemented with 10% FBS. HT-29 cells were cultured in McCoy's 5A medium (Invitrogen) supplemented with 10% FBS. The human lung cancer cell lines H1299 and H460 were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FBS. A549 cells were cultured in F12K (Invitrogen) supplemented with 10% FBS. All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained at 37℃ in an atmosphere of 5% CO_2, 95% air.

An anti-c-MYC (Catalog No. ab32072) antibody

was purchased from Abcam (Cambridge, MA, USA). An anti-FBXW7 (Catalog No. A301-720A) antibody was purchased from Bethyl Laboratories, Inc. (Montgomery, TX, USA). Anti-HA (Catalog No. M20003) and anti-Flag (Catalog No. M20008) antibodies were purchased from Abmart (Shanghai, China). The anti-actin (Catalog No. CW0281) antibody was purchased from CWBiotech (Beijing, China).

1.3 Plasmids, miRNA mimics and inhibitors

To construct a miR-92b overexpression vector, a genomic fragment containing the pre-miR-92b stem loop and the upstream and downstream flanking sequences was cloned from the HEK 293T genome by KOD DNA polymerase (TOYOBO, Osaka, Japan) and inserted into the pCDH lentivector. The following primers were used: miR-92b-*Bgl* Ⅱ-S, 5′ ATATCAGATCTCCAACTCCCCAGCGCG 3′; miR-92b-*Eco*R Ⅰ-AS, 5′ CGCTAGAATTCGACCCACTTACTCACGGTGGC 3′.

The miR-92b promoter (–1500 bp/0 bp) was cloned from HEK 293T genomic DNA and inserted into the pGL3-Basic vector (Clontech, Mountain View, CA, USA). Two primers containing *Xho* Ⅰ and *Hin*d Ⅲ restriction enzyme sites, respectively, were used, and their sequences are as follows: primer –1500, 5′ CCCGGGCTCGAGCCTTGCGGGGCTGGGCC-GCG 3′; primer –0, 5′ CGGTGGGGAGCGGGATCCAAGCTTGGCAT 3′.

The wild-type 3′ UTR of the FBXW7 mRNA was amplified from HeLa cDNA and inserted into the *Pme* Ⅰ-*Sal* Ⅰ sites of the pmirGLO luciferase reporter vector (Promega, Madison, WI, USA). A mutant 3′ UTR of FBXW7 was also generated by PCR. The primers used were as follow: FBXW7-wtUTR-S, 5′ AGCTTTGTTTAAACACCTAGTCACATTGGAGAGTGGGGGGAG 3′; FBXW7-wtUTR-AS, 5′ ACGCGTCGACACTTCAGTGGAAGAAACAGGCATACTAACATG 3′; FBXW7-mutUTR-AS, 5′ ACGCGTCGACTAAGTTATAAGAAAATAAAATTTCTAAGTGAATCAAACCATAGACAC 3′. All constructs were confirmed by DNA sequencing.

For transient overexpression and inhibition of miRNAs, miR-92b mimics(mirVana™ hsa-miR-92b-3p mimics, Cat. # 4464066, Ambion, Austin, TX, USA) and miR-92b inhibitors (micrOFF™ hsa-miR-92b-3p inhibitors, miR20003218, RiboBio, Guangzhou, China), respectively, were transfected to HEK 293T cells by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

1.4 Lentiviral packaging and infection

The pCDH-CMV-MCS-EF1-Puro, pΔ8.2 and pVSVG 3-plasmid system was used for lentivirus packaging. HEK 293T cells were transfected with calcium phosphate using the standard protocol. For stable transfection, cells were infected with lentiviruses and were screened by incubation with 1～2 mg/L puromycin beginning at 48 h post-infection.

1.5 Cell viability assay

Cell viability was assessed by the MTT colorimetric assay. SW620 cells were seeded at 5 000 cells per well in 96-well plates. At different time points (0, 1, 2, 3, 4 and 5 days), 20 μl of MTT (Sigma-Aldrich, St. Louis, MO, USA) solution (5 g/L) was added to each well. After incubation for 4 h at 37℃, the medium was replaced with 150 μl of DMSO, and the plate was allowed to shake on a plate shaker for 15 min. Absorbance at 595 nm and 650 nm were measured using a microplate reader (Thermo Scientific, Waltham, MA, USA). The assay was repeated three times with six replicates per experiment. Values are expressed as fold changes relative to the Day-0 group.

1.6 Xenograft tumor model

Animal experiments were performed according to the SIBS (Shanghai Institutes for Biological Sciences) Guideline for the Care and Use of Laboratory Animals. Four-week-old female BALB/c nude mice were purchased from the Shanghai Experimental Animal Center (Shanghai, China). To establish xenograft tumors, 2×10^6 SW620 cells stably expressing empty vector (EV) or miR-92b were resuspended in 100 μl PBS and subcutaneously injected into the right flank of each mouse (eight mice per group). After about one week, tumor growth was monitored and measured with a Vernier caliper every 3 days. Tumor volume (mm^3) was calculated by length (mm) × width (mm) × width (mm)/2.

1.7 Luciferase reporter assay

Cells in 48-well plates were transfected in quintuple using Lipofectamine 2000. For the miR-92b promoter assay, the pGL3-miR-92b promoter, pcDNA3-c-MYC and the *Renilla* luciferase plasmids (Promega) were co-transfected into HEK 293T cells

for 24 h with 50 ng, 100 ng and 1 ng, respectively. For the FBXW7-3′ UTR assay, HEK 293T cells were co-transfected with 100 ng pmirGLO-FBXW7-3′UTR plasmid and 40 nmol/L of miR-92b mimic for 24 h. Luciferase assays were performed using a dual luciferase assay system (Promega). Relative luciferase activity was determined as the ratio to *Renilla* luciferase activity.

1.8 Real-time quantitative reverse transcription-PCR (quantitative RT-PCR)

Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instruction. The ReverTra Ace qPCR RT kit (TOYOBO) was used to detect and quantify mRNA, and the Mir-X™ miRNA First Strand Synthesis kit (Clontech) was utilized to synthesize first-strand cDNA for detection and quantification of miRNA. Quantitative PCR was performed using SuperReal PreMix Plus SYBR Green (Tiangen, Beijing, China) on a Bio-Rad CFX96 real-time PCR system (Bio-Rad). The following primers were used: c-MYC-F (5′ CCTGGTGCTCCATGAGGAGAC 3′) and c-MYC-R (5′ CAGACTCTGACCTTTTGCCAGG 3′); FBXW7-F (5′ GTGATAGAACCCCAGTTTCA 3′) and FBXW7-R (5′ CTTCAGCCAAAATTCTCCAG 3′); GAPDH-F (5′ GTCTCCTCTGACTTCAACAGCG 3′) and GAPDH-R(5′ ACCACCCTGTTGCTGTAGCCAA 3′); miR-92b-F (5′ ATTGCACTCGTCCCGGCCTCC 3′). GAPDH and U6 were used as internal controls for mRNA and miRNA analysis, respectively. Data are expressed as the fold changes relative to the control group and were calculated based on the following formula: $RQ = 2^{-\Delta\Delta Ct}$.

For the detection of secreted miRNA from serum or culture medium, the MaxRecovery™ BiooPure™ RNA Isolation Reagent (Bioo Scientific, Austin, TX, USA) was used to enrich small RNAs. The remaining procedures were identical to miRNA quantification from cells.

1.9 Western blot analysis

Whole cell lysates were prepared using immunoprecipitation lysis buffer (Beyotime, Jiangsu, China) containing the protease inhibitor PMSF (Beyotime). Total protein was quantified by the Lowry-based *DC* protein assay (Bio-Rad, Hercules, CA, USA) and denatured at 100 ℃ for 5 min. The samples were then size-fractionated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat milk and incubated with primary antibody overnight at 4 ℃, followed by incubation with HRP-linked secondary antibody at room temperature for 1 h. After incubation with chemiluminescence reagents, signals were visualized using a Fujifilm Luminescent Image Analyzer LAS4000 System (Tokyo, Japan). Quantitative analysis was performed using Gel-Pro Analyzer Software.

1.10 Statistical analysis

All the data are shown as the mean ± S.E.M. Comparison between two groups was performed by two-tailed Student's *t*-test using GraphPad Prism (San Diego, CA, USA). Differences were considered statistically significant with a $P < 0.05$.

2 Results

2.1 miR-92b promotes colorectal cancer cell growth in vitro and in vivo

Previous studies showed that miR-92b functions as an oncogene in several types of cancers, such as different types of brain cancer[13] and lung cancer[15, 21]. To investigate its role in colorectal cancer, we collected several patient samples and examined the levels of miR-92b in cancerous tissues and matched non-cancerous tissues. The miR-92b level was significantly higher in tumor samples compared with normal samples, suggesting that miR-92b may potentially function as a tumor-promoting gene in CRC (Figure 1a). In addition, the endogenous miR-92b levels in several colorectal cancer cell lines (SW620, LS174T, LoVo) were higher than the embryonic renal cell HEK 293T (Figure 1b). To demonstrate the role of miR-92b in CRC cells, according to other reported protocol on the constructs of miRNA expression system[22], we stably overexpressed miR-92b in SW620 cells with 6-fold higher expression than the control group, and observed significantly increased cell growth (Figure 1d, e). To determine whether miR-92b promotes tumor cell growth *in vivo*, a xenograft tumor model was tested. SW620 cells stably expressing miR-92b were injected into nude mice, and the tumor growth was monitored over 13 weeks. The results revealed that miR-92b can accelerate the tumor growth *in vivo* (Figure 1f). These results demonstrate that miR-92b promotes colorectal cancer growth both *in vitro* and *in vivo*.

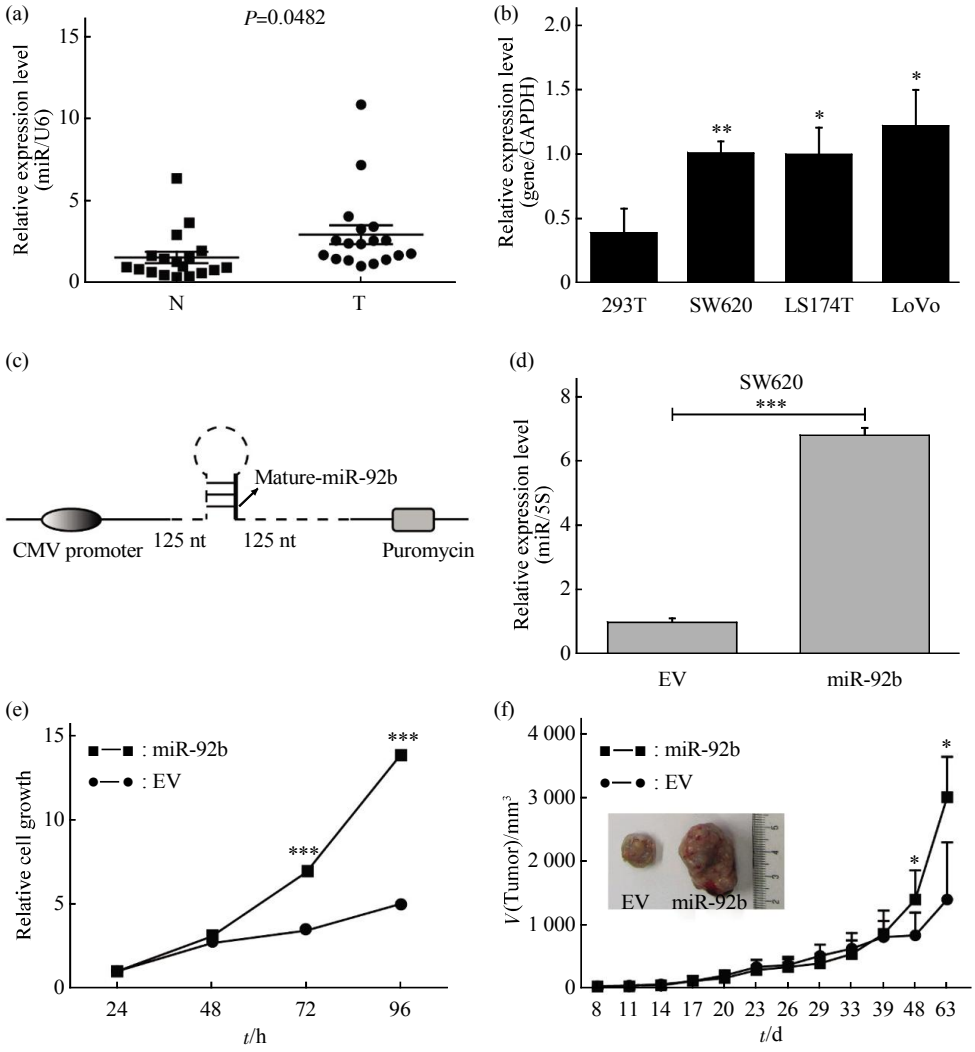


Fig. 1 miR-92b promotes the growth of CRC cells both in vitro and in vivo

(a) Quantitative RT-PCR detection of miR-92b levels in 20 pairs of CRC tissues (T) compared with the adjacent normal tissues (N). (b) Quantitative RT-PCR detection of endogenous miR-92b levels in HEK 293T cells and several colorectal cancer cell lines (SW620, LS174T, LoVo). *$P < 0.05$, **$P < 0.01$ versus the 293T group. (c) Schematic diagram for the construction of the miR-92b overexpression pCDH lentivector. The 22 nt mature miR-92b is shown in bold, and the 125 nt of genomic sequence flanking each side of the miRNA are shown as dotted lines. The constitutive expression of miR-92b was driven by the CMV (cytomegalovirus) promoter, and puromycin was used as a selection marker. (d) Examination of stable miR-92b overexpression efficiency in SW620 cells by quantitative RT-PCR. Values were normalized to 5S RNA and relative to the empty vector (EV) group. (e) Cell viabilities of SW620 cells stably expressing miR-92b were examined by MTT assays. (f) Xenograft tumor growth of subcutaneously injected SW620 cells stably expressing miR-92b. Representative photos of tumors in each group are shown as the inserted image. *$P < 0.05$, ***$P < 0.001$.

2.2 miR-92b is actively secreted in vitro and in vivo

Recent studies reported that some miRNA families can be detected in the plasma and serum [23]. This suggests that miRNAs may function extracellularly. To explore whether miR-92b can be secreted from the cells, we examined the miR-92b levels in the medium of several cancer cell lines, including colorectal cancer cell lines (SW620, HT-29) and lung cancer cell lines (H1299, A549, H460). It was found that miR-92b levels were significantly higher in the medium cultured with the cancer cell lines than in normal medium (Figure 2a ～e). Using miR-221 as a secretory miRNA positive control in the serum of CRC [24], the xenograft tumor model also showed that miR-92b could be secreted into the peripheral blood (Figure 2f). These data suggest that miR-92b functions not only inside the cells but also outside the cells in an actively secreted form.

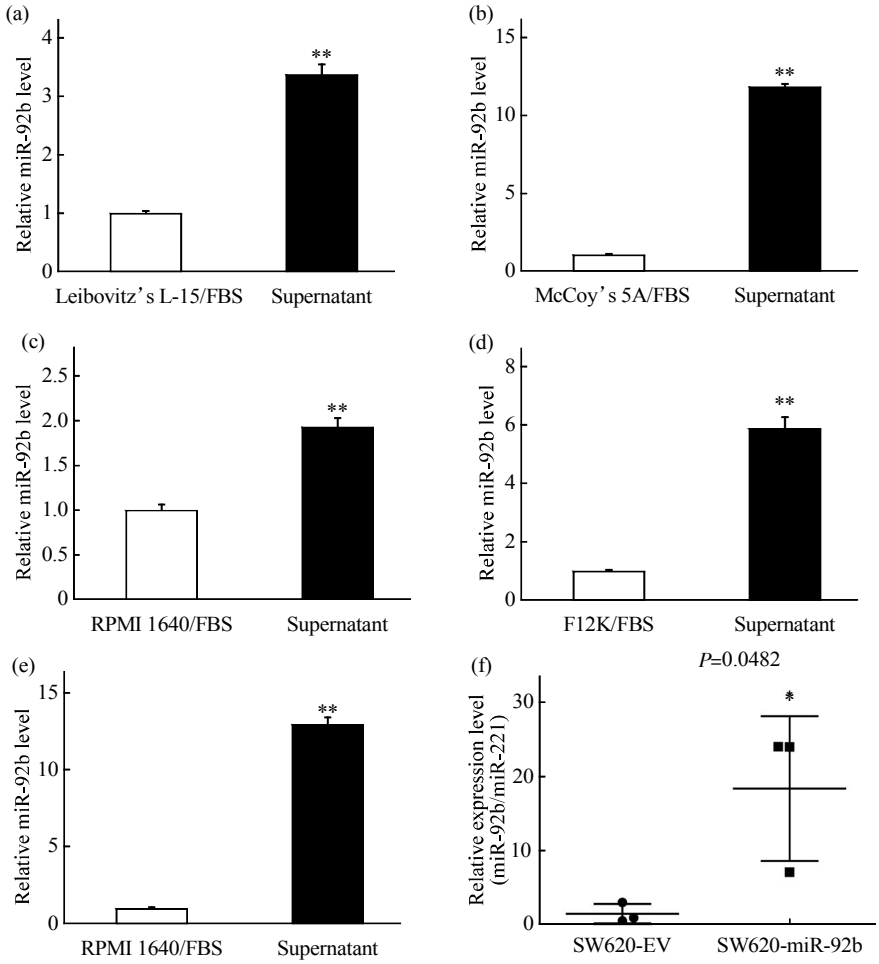


Fig. 2 miR-92b is actively secreted in vitro and in vivo

(a～e) Quantitative RT-PCR detection of miR-92b levels in the culture medium of the colorectal cancer cell lines SW620 (a) and HT-29 (b) and the lung cancer cell lines H1299 (c), A549 (d) and H460 (e) compared to the corresponding uncultured medium with 10% FBS. (f) Quantitative RT-PCR detection of serum miR-92b levels in the peripheral blood of xenograft tumor mice normalized to the miR-221. $*P < 0.05$, $**P < 0.01$.

2.3 c-MYC is responsible for the transcription of miR-92b

We previously demonstrated that miR-92b expression was higher in CRC tumor tissues, but the underlying regulatory mechanism is still unclear. Using the online ConSite prediction program, we identified three consensus E-box sequences (CACGTG), which are canonical c-MYC binding sites, distributed in the promoter region of miR-92b (Figure 3a). Quantitative RT-PCR showed that miR-92b expression was induced by overexpression of c-MYC in HEK 293T cells (Figure 3b) and was decreased by silencing c-MYC in SW620 cells(Figure 3c). To further confirm that miR-92b is a direct target of c-MYC, we performed luciferase reporter assays. miR-92b promoter activity was clearly increased by c-MYC (Figure 3d). In contrast, deletion of the three E-box sequences dramatically reduced the effect of c-MYC on miR-92b promoter activity (Figure 3d). Consistent with the literature, we found that c-MYC levels were higher in colorectal cancer patient samples than in adjacent tissues (Figure 3e). The elevated c-MYC levels coincided with high miR-92b expression in colorectal cancer samples (Figure 1b). These results suggest that miR-92b expression is directly regulated by c-MYC at the transcriptional level.

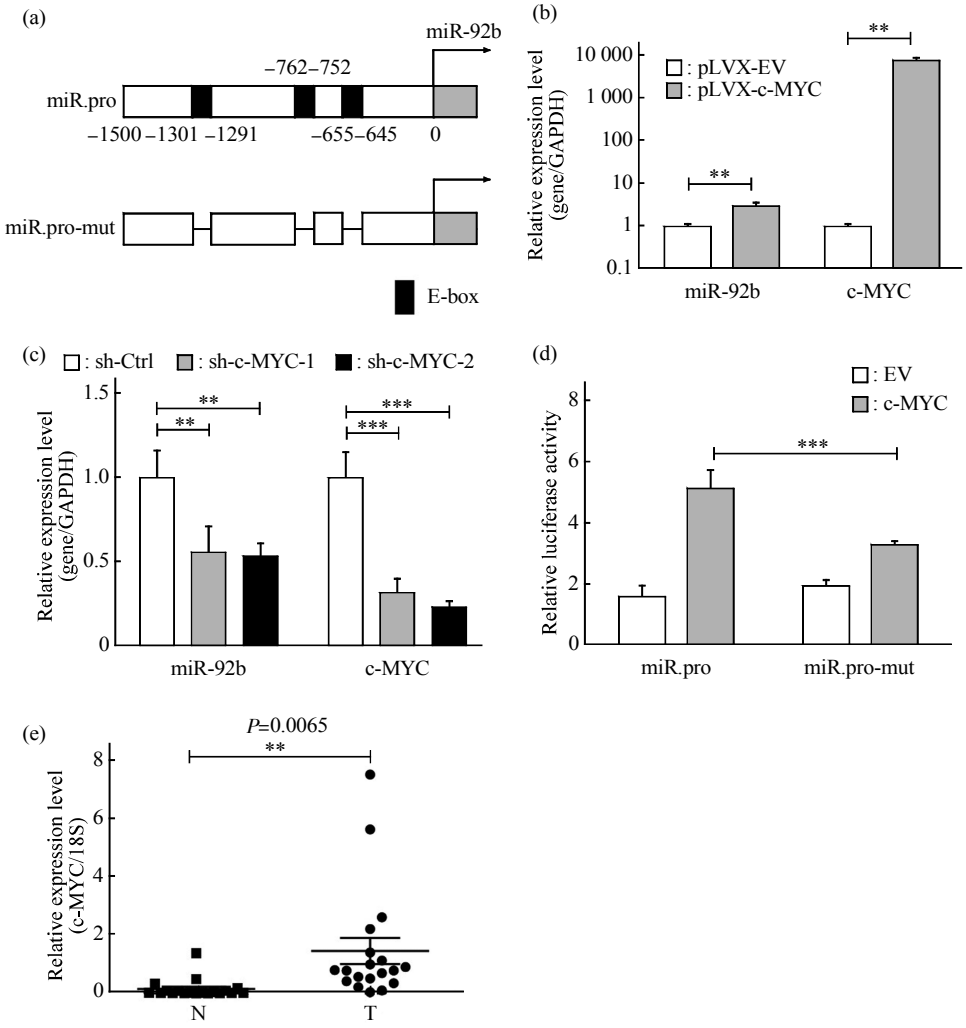


Fig. 3 c-MYC is responsible for the transcription of miR-92b

(a) Schematic diagram of the E-box sequences in the miR-92b promoter predicted by ConSite. (b) Quantitative RT-PCR analysis of miR-92b and c-MYC levels in HEK 293T cells transfected with pLVX-c-MYC for 24 h. (c) Quantitative RT-PCR detection of miR-92b and c-MYC levels in SW620 cells transfected with c-MYC shRNA for 48 h. (d) HEK 293T cells were cotransfected with a miR-92b promoter reporter plasmid and pLVX-c-MYC for 24 h. Luciferase activity was measured. (e) Quantitative RT-PCR detection of c-MYC levels in 20 pairs of CRC tissues (T) compared with the corresponding normal tissues (N). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

2.4 FBXW7 is a new direct target of miR-92b

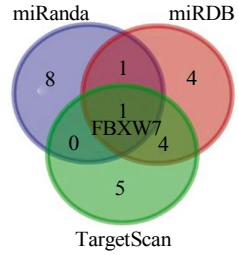
Several tumor suppressor genes were reported to be targets of miR-92b, including DKK3[25] and PTEN[21]. By comparing and merging the top 10 candidates from three different online miRNA target prediction databases (miRanda, miRDB, TargetScan), we found that FBXW7 was a unique predicted target of miR-92b (Figure 4a), although it was also confirmed as a target of miR-92a[26]. There are two miR-92b binding sites in the 3′UTR region of FBXW7 mRNA (Figure 4b). We cloned the 3′ UTR of FBXW7 into the pmirGLO luciferase reporter vector and found that miR-92b mimics effectively decreased FBXW7 3′UTR activity. This effect was completely abolished when the miR-92b binding sites were mutated (Figure 4c). In addition, the FBXW7 protein coding sequence with or without the 3′ UTR of the FBXW7 mRNA was overexpressed in HEK 293T cells. As expected, only the exogenous FBXW7 CDS with the 3′ UTR was significantly decreased by miR-92b (Figure 4d). We further found that the endogenous FBXW7 protein level was also decreased by miR-92b mimics in HEK 293T cells (Figure 4e) and increased by miR-92b inhibitors in SW620 cells (Figure 4f). Collectively, our

data suggest that FBXW7 is a direct target of miR-92b.

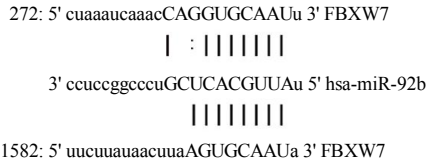
Previous work showed that FBXW7 is a major E3 ligase of c-MYC [18, 27-28]. Our data showed that overexpression of FBXW7 decreased the c-MYC protein level (Figure 4d), which is consistent with previous reports. Here, we found FBXW7 was a target of miR-92b, which suggests that c-MYC might be able to decrease the FBXW7 protein level through miR-92b. Immunoblotting results showed that overexpression of c-MYC greatly decreased the FBXW7 protein level (Figure 4g). Knockdown of c-MYC by shRNA interference increased FBXW7 protein levels in SW620 cells (Figure 4h). These data suggest that a regulatory axis exists among c-MYC, miR-92b and FBXW7 (Figure 4i).

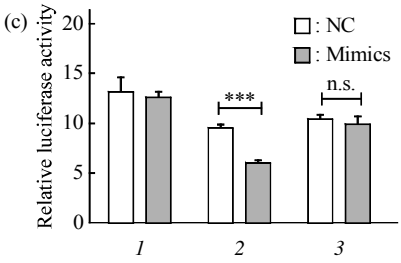
(a)

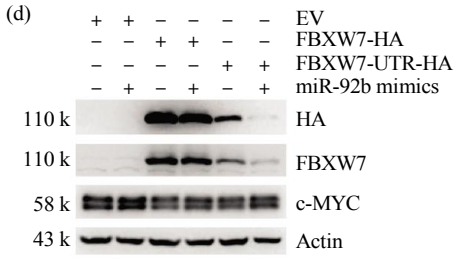
miRanda	*miRDB*	*TargetScan*
PGBD2	CD69	CD69
ERGIC2	SLC12A5	FNIP1
FBXW7	USP28	SLC12A5
TOB1	DCAF6	MAN2A1
UGP2	RAB23	ACTC1
CNOT6L	FBXW7	FBXW7
ZFC3H1	FNIP1	ASPH
MYCBP2	SNX13	DCAF6
KAT2B	FMR1	SYN2
USP28	MAP2K4	EFR3A

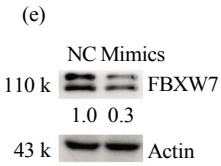


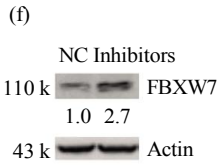
(b)

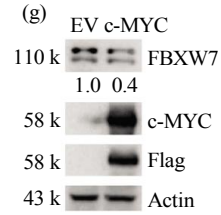


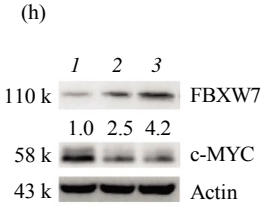












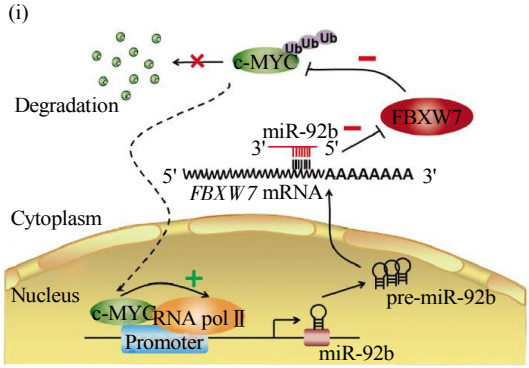


Fig. 4　FBXW7 is a new direct target of miR-92b

(a) The top 10 predicted targets from each of the three online databases are listed. FBXW7 was determined to be a unique target by comparing and merging the target lists. (b) Sequence alignment of miR-92b and its two target sites in the 3′UTR of the FBXW7 mRNA. (c) HEK 293T cells were cotransfected with miR-92b mimics or negative control (NC) with wild-type (wt) or mutant (mut) FBXW7-3′UTR. Relative luciferase activity was measured 24 h post-transfection. ***$P < 0.001$, n.s., not significant. *1*: EV; *2*: FBXW7-3′UTR-wt; *3*: FBXW7-3′UTR-mut. (d) HEK 293T cells were transfected with miR-92b mimics or the FBXW7 expression plasmid with or without the 3′UTR alone or combined. FBXW7 and c-MYC protein levels were analyzed by Western blotting 48 h post-transfection. (e, f) Endogenous FBXW7 protein levels were analyzed by Western blots in HEK 293T cells transfected with miR-92b mimics (e) or SW620 cells transfected with miR-92b inhibitors (f) for 48 h. (g, h) Western blot detection of FBXW7 in HEK 293T cells transfected with the c-MYC expression plasmid (g) or SW620 cells transfected with a c-MYC shRNA plasmid (h) for 48 h. *1*: sh-Ctrl; *2*: sh-c-MYC-1; *3*: sh-c-MYC-2. (i) The feedback regulatory model includes miR-92b, c-MYC and FBXW7. c-MYC promotes the transcription of miR-92b by direct binding to its promoter. miR-92b represses FBXW7 expression by targeting the 3′UTR of *FBXW7* mRNA. As FBXW7 is a major E3 ligase of c-MYC, miR-92b might form a feedback loop with c-MYC and FBXW7.

3 Discussion

miR-92b is highly expressed in several types of cancers. However, how miR-92b is regulated still remains unclear. Here, we observed that miR-92b had relatively higher expression levels in CRC compared to the adjacent tissues. The increased miR-92b promoted CRC cell viability *in vitro* and xenograft tumor growth *in vivo*. Additionally, we found that miR-92b secretion was increased by CRC cells both in cultured medium and the peripheral blood of xenograft mice. c-MYC could directly promote the transcription of miR-92b, regulating the protein levels of its E3 ligase FBXW7 by promoting direct binding of miR-92b to the 3′ UTR of FBXW7 mRNA. Our findings suggest that c-MYC-regulated miR-92b up-regulation contributes to CRC tumorigenesis and disease progression and identify miR-92b as a potential therapeutic target and CRC diagnostic biomarker.

Studies have increasingly demonstrated the oncogenic potential of miR-92b [29]. Previous research has identified several miR-92b targets, which were primarily tumor suppressors. Through targeting these tumor suppressors, such as PTEN [21] and DKK3 [25], miR-92b is able to promote cell proliferation and migration[15]. However, few studies have focused on the characteristics and the upstream signaling of miR-92b. Here, we found that miR-92b can be actively secreted outside the cells both *in vitro* and *in vivo*. We also provided evidence for the positive regulatory effect of c-MYC on miR-92b.

To our knowledge, this is the first report showing that miR-92b is directly regulated by c-MYC on its promoter activity in CRC cancer cells. It was demonstrated that c-MYC induces miR-92b expression by binding to the three canonical E-box sites in the miR-92b promoter region (Figure 3a～d). c-MYC is overexpressed in most cancers, including CRC [30]. Consistent with the higher expression of miR-92b in samples from colorectal cancer patients, we also found that c-MYC was highly expressed in our samples from colorectal cancer patients. This may be one way by which miR-92b is up-regulated in CRC cells and perhaps other cancer cell types as well.

In this study, we also identified FBXW7 as a new miR-92b target. FBXW7 is an E3 ligase that targets c-MYC, cyclin E, NOTCH and JUN [31]. Loss of FBXW7 would result in elevated levels of these substrates, resulting in deregulation of cell proliferation to further promote tumorigenesis. Down-regulation of FBXW7 may be involved in the cell growth induced by miR-92b. Interestingly, our data showed that c-MYC can down-regulate FBXW7 through induction of miR-92b(Figure 4e). Collectively, to some degree, it suggests that c-MYC/miR-92b/FBXW7 is a positive regulatory axis.

To date, only a few miRNA families have been reported to be selectively enriched in the extracellular fractions [32]. These secreted miRNAs are reported to have significant effects on tumorigenesis [33]. Secreted miRNAs can exist in a stable and extracellular form. They can be easily used as blood-based diagnostic and prognostic markers and for therapeutic approaches. Our data showed that miR-92b can be actively secreted outside the host cell. Considering the role of miR-92b in tumor cell growth (Figure 1d, e) and metastasis[14-15], miR-92b may be used as a candidate serum marker for cancer diagnosis.

However, due to the lack of CRC patient serum samples, we have not been able to evaluate the miR-92b levels in CRC patients' serum. Additionally, further validations in large cohorts are necessary to confirm the correlation between miR-92b and c-MYC expression levels. Identification of an easily detected biomarker is very useful for cancer diagnosis. Body fluid is easy to obtain from cancer patients for diagnostic purposes. Our data demonstrate that miR-92b might be a potential candidate for blood-based cancer diagnosis. Given the ability of miR-92b to subvert c-MYC deregulation by FBXW7, miR-92b should also be considered a potential therapeutic target in the treatment of CRC.

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c-MYC 调节的 miRNA-92b 在结直肠癌发生发展中的功能及机制探究 *

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摘要　microRNAs(miRNAs)在参与癌症发生、发展过程中起着十分重要的作用．目前，miR-92b 在结直肠癌中的作用及相关机制还未见报道．本研究探讨了 miR-92b 在结直肠癌发生发展中的功能及潜在机制．采用 RT-qPCR 方法发现，miR-92b 在人结直肠癌临床样本中与癌旁组织相比显著高表达．通过结肠癌细胞株 SW620 稳转细胞及裸鼠皮下成瘤模型，发现过表达 miR-92b 可以显著促进细胞增殖及体内肿瘤生长．同时还发现 miR-92b 可以分泌形式存在于胞外及外周血中，提示 miR-92b 是一个具有分泌特性的 microRNA．在分子机理方面，c-MYC 可通过调节 miR-92b 的启动子活性从而促进后者转录，并且 c-MYC 在结直肠癌组织样本中也存在高表达．进一步，通过在线预测、报告质粒活性检测及蛋白质印迹技术证实 FBXW7 是一个新的 miR-92b 靶基因．由于 FBXW7 已报道为 c-MYC 泛素降解过程中的关键泛素化连接酶之一，本研究结果提示结直肠癌中 c-MYC、miR-92b 及 FBXW7 三者间可能存在分子调节环路．综上所述，本研究为 miR-92b 在结直肠癌中的功能及机制提供了新的视角，并为 miR-92b在结直肠癌早期诊断中的应用提供了新的参考．

关键词　miR-92b，结直肠癌，分泌型 microRNA，c-MYC，FBXW7

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