An oncogenic role of miR-592 in tumorigenesis of human colorectal cancer by targeting Forkhead Box O3A (FoxO3A)

Qi Fu\textsuperscript{a,b,c}, Yong Du\textsuperscript{a,b*}, Chun Yang\textsuperscript{b}, Dong Zhang\textsuperscript{b}, Ningmei Zhang\textsuperscript{d}, Xiaoming Liu\textsuperscript{a}, William C. Cho\textsuperscript{e} and Yinxue Yang\textsuperscript{a,b}

\textsuperscript{a}Human Stem Cell Institute, General Hospital of Ningxia Medical University, Yinchuan, China; \textsuperscript{b}Department of Colorectal Surgery, General Hospital of Ningxia Medical University, Yinchuan, China; \textsuperscript{c}Graduate School, Ningxia Medical University, Yinchuan, China; \textsuperscript{d}Department of Pathology, General Hospital of Ningxia Medical University, Yinchuan, China; \textsuperscript{e}Department of Clinical Oncology, Queen Elizabeth Hospital, Kowloon, Hong Kong

\textbf{ABSTRACT}

\textbf{Objective:} A microRNA (miRNA) that functionally downregulates the expression of tumor suppressors can be defined as an oncomir. Here, we interrogate the biological significance of miR-592 in colorectal cancer (CRC).

\textbf{Research design and methods:} The expression of miR-592 in CRC tissues and cell lines was ascertained by qRT-PCR assay, and the expression of its target gene was determined by immunohistochemistry staining. The oncogenic role of miR-592 was assessed in terms of cell proliferation, migration, and clonogenicity \textit{in vitro}, whereas the tumorigenicity was assessed by inhibiting endogenous miR-592 in CRC cells \textit{in vivo}.

\textbf{Results:} A striking upregulation of miR-592 was observed in CRC tissues and cell lines compared to the matched adjacent non-tumor tissues and normal colon cells. Importantly, Forkhead Box O3A (FoxO3A) was identified as a novel target of miR-592. miR-592 inhibitor exhibited a significant reduction of migration, proliferation, and clonogenicity in CRC cells. These cells also displayed a decreased tumorigenicity in SCID mice relative to the control cells.

\textbf{Conclusion:} These data suggest that miR-592 may promote the progression and metastasis, in part, by targeting FoxO3A in CRC. miR-592 may be a novel target for CRC treatment and antiagomir-592 may inhibit the proliferation and metastasis of CRC cells.

1. Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers worldwide. Despite the pathogenesis of CRC has been well-characterized, the reasons for the high relapse rate of this type of cancer are not fully understood.\cite{1} Recently, accumulating evidence has demonstrated the importance of microRNA (miRNA), a family of small noncoding RNAs that negatively regulate the gene expressions of target mRNAs at the posttranscriptional level, have a great implication in cancer development and progression through a nongenetically mutational mechanism. Dysregulations of miRNAs have been involved in the initiation, growth, metastasis, recurrence, and acquisition of drug-resistance in a variety of cancers.\cite{2-5} Indeed, recent studies have demonstrated that alterations of miRNA expression profiling in tumors compared with adjacent normal tissues have been observed in CRC, which has been proposed to correlate with the stages and survivals in CRC patients, in which miRNAs can exert their regulatory roles by directly targeting genes in the key steps of initial and metastatic processes, and acquired drug-resistance.\cite{6} In this context, miRNAs can play a functionality of either tumor-suppressors (antagomirs) or oncogenes (oncomirs).\cite{7,8}

The Forkhead Box O (FOXO) transcription factor family members are evolutionarily conserved proteins that have recently been highlighted as an important regulator in multiple signaling pathways.\cite{9} To date, FOXO1, FoxO3A, FOXO4, and FOXO6 proteins have been identified in humans. Among them, the FoxO3A is the most extensively investigated protein, owing to its unique and pivotal regulatory roles in cell proliferation, apoptosis, metabolism, oxidative stress, and survival.\cite{10} In this regard, a dysregulated expression of FoxO3A gene has been closely linked to the progression of several types of cancers, particularly in the breast cancer, hepatocarcinoma, glioma, prostate cancer, and CRC, in which FoxO3A exhibits a tumor suppressor role by acting as a regulatory node of signaling networks.\cite{11-18} For instance, FoxO3A could exert its antitumor properties by inducing the expression of proapoptotic genes, or interfering with signaling cascades, such as Wnt/beta-catenin, phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mTOR, or MAPKs pathways in hepatocellular carcinoma family members and could be useful in the treatment of hepatocarcinoma.\cite{19} In addition, a remarkably reduced abundance or loss of FoxO3A was found in metastatic colorectum, primary CRC and liver metastasis,\cite{20} and in high-grade pelvic serous carcinogenesis.\cite{14}
There is a compelling body of evidence suggesting that the expression of FoxO3A can be regulated by miRNAs in several cancers and other diseases.[13,21–26] For example, miR-155 was able to regulate cell survival, growth, and chemosensitivity by targeting the FoxO3A gene in breast cancer [13] and glioma.[25] Other miRNAs, including miR-96,[15,21,26] miR-27a,[22] miR-30d, [24] and miR-194,[27] are also able to target FoxO3A. Vice versa, an alteration of FoxO3A expression is in turn able to modulate the expression of several miRNAs.[16,28] For instance, FoxO3A showed an ability to induce miR-622 expression, which in turn suppresses tumor metastasis by targeting hypoxia-inducible factor-1alpha in extracellular signal-regulated kinase-responsive lung cancer.[11] In another example, FoxO3A was demonstrated to be able to induce miR-34b/c transactivation, which consequently inhibited Wnt/β-catenin signaling by targeting β-catenin and suppressed epithelial-to-mesenchymal transition in prostate cancer cells.[16] Mechanistically, an upregulated oncomir miR-21 was able to lead a downregulation of phosphatase and tensin homolog (PTEN) and sequentially activate PI3K/AKT signaling. An activation of AKT signaling in turn phosphorylated FoxO3A and triggered re-localization of FoxO3A proteins from the nucleus to the cytoplasm. A decreased FoxO3A abundance in nuclear then suppressed the expression of miR-34b and miR-34c in breast cancer cells, and a systemic delivery of miR-34b/c or with antagonomiR-21 significantly inhibited breast tumor growth in vivo.[29] Consistently, an activation of MK5-FoxO3A-miR34b/c pathway also could further increase c-Myc levels in CRC.[30] These studies clearly suggest that miRNA-mediated biological roles in CRC pathogenesis may be through a mechanism in part by modulating FoxO3A signaling.

Recently, we and other groups have uncovered a strikingly upregulated miR-592 in CRC tissues as compared to the matched adjacent non-tumor tissues, which may imply it may play an oncogenic role in CRC tumorigenesis.[31,32] Therefore, we aimed to investigate biological roles of miR-592 in regulation of capacity of CRC cell metastasis and tumorigenesis in the present study, and we identified that FoxO3A mRNA was a novel target of miR-592 in CRC for the first time.

2. Materials and methods

2.1. Ethics statement

Human colorectal tissue was collected with a protocol approved by the Ethics Committee for the Conduct of Human Research at Ningxia Medical University. Written consents were obtained from each individual according to the Ethics Committee for the Conduct of Human Research protocol. All participants were over 18 years of age and provided written informed consent for the publication of the data. The Human Research Ethics Committee at Ningxia Medical University approved this study.

2.2. Human colorectal tissue samples and cell lines

Fifty tumor samples with histologic evidence of CRC and matched adjacent non-tumor tissues were archival samples from department of Medical Pathology Department, General Hospital of Ningxia Medical University from the year of 2014.

### Table 1. Demographics and relative miR-592 expression in tumor tissues compared to the matched adjacent non-tumor tissues of various stages of colorectal cancer.

| Subject | Age | Gender | Degree of differentiation | TNM stage | Lymph node metastasis | Relative miR-592 expression$^1$ |
|---------|-----|--------|---------------------------|-----------|-----------------------|-------------------------------|
| CRC01   | 64  | M      | Medium-low                | II        | No                    | 6.71                          |
| CRC02   | 74  | M      | Medium-low                | II        | No                    | 2.01                          |
| CRC03   | 56  | F      | Medium-low                | III       | Yes                   | 1.39                          |
| CRC04   | 63  | M      | Medium-low                | III       | Yes                   | 0.45                          |
| CRC05   | 75  | F      | Medium-low                | IV        | Yes                   | 0.08                          |
| CRC06   | 68  | M      | Medium-low                | II        | No                    | 1.87                          |
| CRC07   | 47  | F      | Medium-low                | III       | Yes                   | 3.04                          |
| CRC08   | 64  | M      | High                      | II        | No                    | 1.40                          |
| CRC09   | 75  | M      | High                      | II        | No                    | 0.74                          |
| CRC10   | 62  | F      | High                      | III       | Yes                   | 5.30                          |
| CRC11   | 57  | M      | High                      | III       | Yes                   | 8.14                          |
| CRC12   | 77  | F      | High                      | II        | No                    | 49.06                         |
| CRC13   | 64  | F      | High                      | IV        | Yes                   | 10.39                         |
| CRC14   | 75  | M      | High                      | III       | Yes                   | 15.31                         |
| CRC15   | 66  | M      | High                      | II        | Yes                   | 9.78                          |
| CRC16   | 57  | M      | Medium                    | II        | No                    | 0.78                          |
| CRC17   | 62  | M      | Medium                    | I         | No                    | 2.75                          |
| CRC18   | 32  | M      | Medium                    | III       | Yes                   | 36.72                         |
| CRC19   | 57  | M      | Medium-high               | II        | No                    | 0.54                          |
| CRC20   | 64  | M      | High                      | II        | No                    | 7.14                          |
| CRC21   | 76  | M      | High                      | II        | No                    | 1.13                          |
| CRC22   | 50  | M      | Medium                    | II        | No                    | 1.98                          |
| CRC23   | 55  | M      | High                      | II        | No                    | 1.29                          |
| CRC24   | 59  | F      | Medium                    | II        | No                    | 2.33                          |
| CRC25   | 61  | F      | MA                        | III       | Yes                   | 3.03                          |
| CRC26   | 74  | M      | Medium                    | II        | No                    | 0.14                          |
| CRC27   | 77  | M      | Medium                    | II        | No                    | 2.19                          |
| CRC28   | 59  | M      | High                      | III       | Yes                   | 2.33                          |
| CRC29   | 47  | F      | Medium-high               | IV        | Yes                   | 17.59                         |
| CRC30   | 61  | M      | Medium-low                | III       | Yes                   | 2.04                          |
| CRC31   | 48  | M      | Medium-high               | II        | No                    | 1.46                          |
| CRC32   | 49  | M      | Medium                    | III       | Yes                   | 61.08                         |
| CRC33   | 63  | F      | Medium-low                | III       | Yes                   | 5.82                          |
| CRC34   | 76  | M      | Medium-low                | II        | No                    | 3.17                          |
| CRC35   | 82  | F      | High                      | IV        | No                    | 4.14                          |
| CRC36   | 47  | M      | Medium-low                | I         | No                    | 0.52                          |
| CRC37   | 62  | M      | Medium                    | I         | No                    | 0.07                          |
| CRC38   | 64  | M      | Medium                    | III       | Yes                   | 1.92                          |
| CRC39   | 82  | F      | Medium                    | II        | No                    | 2.88                          |
| CRC40   | 64  | F      | Medium                    | II        | No                    | 1.89                          |
| CRC41   | 62  | F      | Medium-high               | II        | No                    | 1.18                          |
| CRC42   | 59  | F      | Medium-high               | III       | Yes                   | 23.6                          |
| CRC43   | 46  | F      | Medium-high               | II        | No                    | 3.35                          |
| CRC44   | 59  | M      | Medium                    | II        | No                    | 9.39                          |
| CRC45   | 81  | M      | Medium-high               | II        | Yes                   | 13.9                          |
| CRC46   | 76  | M      | Medium-low                | II        | No                    | 25.5                          |
| CRC47   | 32  | M      | Medium                    | III       | Yes                   | 1.6                           |
| CRC48   | 57  | M      | Medium-high               | I         | No                    | 0.71                          |
| CRC49   | 77  | M      | Medium-low                | II        | No                    | 5.63                          |
| CRC50   | 66  | M      | High                      | II        | Yes                   | 13.43                         |

$^1$Fold of miR-592 transcript in tumor tissues over the corresponding adjacent non-tumor tissue.

M: Male; F: female; MA: mucoid adenocarcinoma; dL: linear image density; rt: relative transcript; c: cycle; FBS: fetal bovine serum; DMEM: Dulbecco's modified eagle medium; pen: penicillin; strep: streptomycin.

(Table 1). The pathologic tumor staging was determined according to the International Union Against Cancer (2009). Cell lines of human embryonic kidney 293, human CRC cell lines HCT116, LOVO, LS174T, and SW480, and normal intestinal epithelial cell line CCD-18Co were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured and maintained at 37°C in a humidified atmosphere of 5% CO2 95% air in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% pen/strep.
2.3. Immunohistochemistry staining

The expression of FoxO3A protein in clinic human CRC and matched adjacent non-tumor tissues was evaluated by an immunohistochemistry (IHC) staining using rabbit anti-FoxO3A antibody (1:200 from Abcam, USA). Archival paraffin-embedded sections (5 µm) were deparaffinized and rehydrated through graded alcohol solution, the tissue sections were then microwaved in 10 mM sodium citrate pH 6.0 for 15 min and cooled down to room temperature (RT) for a purpose of antigen retrieval, followed by treating the sections with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) for 15 min to inactivate endogenous peroxidase before they were blocked with blocking buffer (5% donkey serum in PBS) for 2 h at RT. The primary antibody was then applied (1:200 in blocking buffer) on the section and incubated overnight at 4°C. Paralleled sections incubated with normal rabbit IgG was used for negative controls. After washing for 3 × 5 min in PBS, sections were incubated with peroxidase-labeled donkey anti-rabbit IgG (ZSGB-Bio Origene, Beijing, China) (1:500 in blocking buffer) for 30 min at RT. The FoxO3A signal was developed with 3,3′-diaminobenzidine peroxidase substrate, followed by counterstaining with hematoxylin if it was applicable. The stained sections were examined and photographed on a Nikon Optiphot II microscope equipped with a camera. The non-counterstained sections were also randomly imaged using a 10× objective lens for five fields of each section, and five sections for each sample were evaluated. The obtained images were then used for a semi-quantitative analysis of the FoxO3A expression by measuring the integrated absorbance (IA) using image analysis software Image-Pro Plus 6.0 (IPP 6.0, Media Cybernetics, Silver Spring, MD, USA), and the average of the IA values of each sample was used as an index of the expression of FoxO3A expression.[33]

2.4. Experimental validation of miR-592 target

In order to validate that the FoxO3A mRNA was a target of miR-592, a reporter plasmid containing luciferase with the 3′ UTR (untranslated region) sequence of human FoxO3A mRNA was generated. The following primers were designed based on GenBank database (NM_201559) and were used for amplification of wild-type and mutated 3′UTR of FoxO3A mRNA: the sequence of common forward primer was 5′-GGCCGGCTCGAGCAGTGGCCCTTTAATGATCTC-3′, reverse primer for the wild-type of FOXO3 mRNA 3′UTR was 5′-AATGCGGCCCTCCTACCTCTGTGCTCTATG-3′, and the reverse primer for mutated FoxO3A mRNA 3′UTR was 5′-GCGCTCGAGCAGTGGCCCTTTAATGATCTCTTCTTCTCTTTAGACCCCTCCTAATGACTGACACAGCTACAGAGAAAACC-3′. The cDNA generated from LOVO RNA was used as templates for amplification of FoxO3A 3′UTR fragment by a PCR assay. The wild-type and mutated 3′UTR fragment were then cloned into the downstream of luciferase reporter gene of pMIR-Report vector (Invitrogen, Grand Island, NY, USA), by which the respective FoxO3A mRNA luciferase reporter vectors, pMIR-Report/FOXO3 (harboring wild-type 3′UTR) and pMIR-Report/Mut-FOXO3 (containing a mutated 3′UTR), were generated. The specificity of miR-592 targeting FOXO3 mRNA was ascertained by co-transfection of plasmid DNA of pSilcoR/miR-592, miR-592/inhibitor or pSilcoR/NC, and pMIR-Report/FOXO3 or pMIR-Report/Mut-FOXO3 into 293T cells and determined in terms of the relative activity of firefly luciferase unit at 48 h post-transfection using a dual-luciferase Reporter assay kit (Promega, Madison, WI, USA). A Renilla luciferase expressing plasmid pRL-TK (Promega, Madison, WI, USA) was always included in the transfection to normalize the efficiency of each transfection.

2.5. Quantitative reverse transcription-PCR

Total RNA from infected cells was isolated with an RNA Miniprep kit, and small RNA was purified with an RNAiso kit per manufacturer’s recommendations (Takara, Dalian, China). Total RNA from archival paraffin sections was isolated using a Mag-Bind® FFPE RNA Kit (Omega Bio-Tek Norcross, GA, USA). The purified RNA was then used for reverse transcription of the first-strand cDNA synthesis by reverse transcription using M-MLV reverse transcriptase. The expression levels of miRNAs were assessed by the stem-loop reverse transcription-polymerase chain reaction (RT-PCR) method using the Hairpin-it™ miRNAs. The sequence of the primer used for reverse transcription of mature miR-592 included a stem-loop structure, which was designed based on the sequence of miR-592 (Table 2). The sequence of primer set for amplification of miR-592 and U6 promoter was listed in Table 2. The U6 promoter was included and used to normalize for sample loading and RNA abundance. The quantitative reverse transcription-PCR (qRT-PCR) was performed using a Bio-Rad i5Q lightcycler using a Takara SYBR RT-PCR kit (Takara, Dalian, China). Relative expression was calculated in terms of the relative activity of firefly luciferase unit using the ΔΔCt method.[34] The specificity of the primer sets was determined by sequencing the product of each qRT-PCR reaction.

2.6. Generation and infection of lentivector expressing miR-592 inhibitor

In order to suppress endogenous miR-592 in CRC cells, lentivector expressing miR-592 inhibitor or scramble mRNA

### Table 2. The sequences of primers used for reverse transcription and PCR.

| Application       | Primer                  | Sequence (5′→3′)            |
|-------------------|-------------------------|-----------------------------|
| Reverse transcription | miR-592 RT       | GTCGTATCCAGTCGAGGTCCGAGGTATTTGCCACTGGGATACGCAGACTCTCA |
|                   | U6 RT                  | AACGCCTCAGAATTTGGCT         |
|                   | Forward                | AGCTGGTCAATACGCCATGA        |
|                   | Reverse                | GTGAGAGTCCGAGTGG           |
|                   | U6 promoter forward    | CCTGCATTGGCAGCACA          |
|                   | U6 promoter reverse    | AAGCCTCAGAATTTGGCT         |

qRT-PCR: Quantitative reverse transcription-PCR.
was generated by Shanghai GeneMarker Inc (Shanghai, China), which was designated as LV-mir-592-inh (core sequence: 5’-ACATCATCGCATATTGACACAA and LV-NC (core sequence: 5’-GTGGAATCTCGTCGACGTTTAAGAGACGTGACACGT-3’) respectively. For construction of the lentiviral vector expressing miR-592-inh, oligonucleotides of sense strand (5’-ATGCGAATTCAAAAAAGTTGTCTAACCTCACCTGTTCTCTTGAAACAGGTGACACGT-3’) and antisense strand (5’-ATGCGAATTCAAAAAAGTTGTCTAACCTCACCTGTTCTCTTGAAACAGGTGACACGT-3’) were synthesized, which were based on the sequence of human miR-592 from miRBase database. Restriction endonuclease HpaI and XhoI were introduced at 5’-ends of these oligonucleotides, respectively. The mixture of the sense and antisense oligonucleotides was then used for production of the precursor of small hairpin RNA of miR-592 inhibitor by temperature annealing approach. miR-592 inhibitor precursor was modified with appropriate restriction enzymes and cloned into a miRNA expressing plasmid, pSicoR (Department of Biological Chemistry, School of Medicine, Fudan University, Shanghai, China), to generate the vector expressing miR-592 inhibitor, which was designated as pSicoR/miR-592oinh in this study. Using the same approach, a negative control vector (pSicoR/NC) was also generated using oligonucleotides of 5’-ATGCGAATTCAAAAAAGTTGTCTAACCTCACCTGTTCTCTTGAAACAGGTGACACGT-3’ for sense strand and 5’-ATGCGAATTCAAAAAAGTTGTCTAACCTCACCTGTTCTCTTGAAACAGGTGACACGT-3’ for antisense strand. For production of the lentiviral LV-mir-592-inh or LV-NC vectors, 1 x 10^8 HEK 293T cells were seeded per well in six well plates with 2 mL of DMEM/10% FBS without antibiotics. The next day, the medium was replaced with 1 mL DMEM without FBS and antibiotics. Subsequently, the pSicoR/miR-592-inh vector (1.5 μg) (for LV-mir-592-inh) or pSicoR/NC (for LV-NC) (1.5 μg) was co-transfected with packaging plasmids pCMV-VSV-G (0.5 μg) and pCMV-dR8.91 (1.0 μg) (Department of Biological Chemistry, School of Medicine, Fudan University, Shanghai, China) with TransLipid Transfection Reagent as suggested by the manufacturer. The medium was replaced with 2 mL of DMEM/10% FBS at 6 h post-transfection. The supernatant was harvested at 48 h after transfection, followed by being filtered through a 0.45-μm pore size low protein binding filter, and then concentrated to 1/100 volume by ultracentrifugation with 50,000 x g at 4°C for 2.5 h using a SW28 rotor (Beckman Coulter, Fullerton, CA, USA) and Sorvall Ultra 80 (Kendro Laboratory Products, Newtown, CT, USA). The virus particle pellet was resuspended in 4% β-lactose in PBS and frozen at −80°C till use. The viral particles were titered in 293T cells by counting EGFP-positive cells, CRC LOVO and LS174T cells were, respectively, infected with each of above viral vectors and continuously cultured for 3 days before being used, or screened in media containing 2.5 μg/mL puromycin for additional 1 week to remove un-transduced cells, the cell pools were then collected and employed for further studies.

2.7. Western blotting analysis

Whole-cell lysates (75.0 μg) or homogenized tissues were prepared in a lysis buffer (50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40) and were resolved by a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel, followed by being transferred to a PVDF membrane (Millipore, USA). The membranes were probed with mouse anti-FoxO3A antibody (Abcam, USA) and anti-beta-actin antibody (Boster, Wuhan, China) for the interested protein FoxO3A and endogenous beta-actin for loading control, respectively. The blots were developed using the enhanced chemiluminescence reagent (Advansta, Menlo Park, CA, USA) after they were incubated with the appropriate peroxidase-labeled secondary antibodies. The levels of protein expression were semi-quantified by optical densitometry using ImageJ Software version 1.46 [http://rsb.info.nih.gov/ij/]. The ratio between the net intensity of each sample divided by the beta-actin internal control was calculated as densitometric arbitrary units which served as an index of relative expression of a protein of interest.[33]

2.8. MTT assay

Cell proliferation was determined by using the MTT cell proliferation kit (Solarbio, Beijing, China). Lentiviral infected LOVO or LS174T cells were split and seeded in each 96-well plates at a density of 2 x 10^4 per well and allowed to adhere overnight. The cells were then used for an MTT assay at indicated time points per the manufacturer’s instruction (Bio-Rad Laboratories, Inc., Irvine, CA, USA).

2.9. Cell scratch assay

The lentiviral infected CRC cells were seeded at 80% confluent and infected with lentiviral vectors for 48 h (cells were grown to confluence) in six well culture plates. The cells were then scratched with 200-μL pipette tips. The resultant unattached cells were removed by washing with pre-warmed PBS for three times, the wounded monolayers were cultured for additional 48 h prior to be stained with 0.1% crystal violet solution. The closure of the wounded areas was observed under a microscope at 40× magnification (Leica, Germany) and photographed. The gap of closure was quantified with the NIH ImageJ image processing program. These experiments were performed in triplicate. Each condition was tested in duplicate and each experiment was repeated for at least three times.

2.10. Clonogenic assay

A clonogenic assay was used for assessing the stemness of CRC cells. For clonogenicity, single cell suspension of lentivirally infected cells were seeded on 35-mm dishes. Cells were continuously cultured for 10 days with refreshment of medium 3-day interval. For colony counting, the medium was removed and the cells were rinsed with PBS prior to be fixed with 4% paraformaldehyde at RT for 5 min. After removing the fixation solution, the cells were then stained with 0.5% crystal violet solution and incubated at RT for 30 min. The staining solution was carefully removed, and the cells were rinsed with H2O to remove residual staining solution before air-drying the plate at RT for up to a day. Count number of colonies and calculate under a light microscope. Each condition was tested in duplicate and each experiment was repeated for three times.
2.11. In vivo analysis of tumorigenetic capacity of CRC cells

The tumorigenic capacity of CRC cells was evaluated in 6–8-week-old female severe combined immunodeficiency (SCID) mice. The mice were subcutaneously inoculated with 200-μL Matrigel (BD) containing $1 \times 10^6$ of CRC cells (LV-NC or LV-miR-592-inh transduced). The animals were euthanized and the tumor tissues of injection sites were collected for evaluation of tumorigenicity after 3 weeks following the cell transplantation. The diameter of tumor was measured and used as an index of the tumorigenesis.

3. Statistical analysis

All data collected in this study were obtained from at least three independent experiments for each condition. SPSS 17.0 analysis software (SPSS Inc., Chicago, IL, USA) and PRISM 5 (GraphPad software, La Jolla, CA, USA) were used for the statistical analysis. Statistical evaluation of the data was performed by one-way ANOVA when more than two groups were compared with a single control and t-test for comparison of differences between the two groups. Significant differences were assigned to $p$ values <0.05, <0.01, and <0.0001 denoted by *, **, and ***, respectively. Data were presented as the mean ± standard deviation.

4. Results

4.1. Upregulated miR-592 in human CRC

By analyzing the miRNA profiling analysis using 10 pairs of CRC tumor tissues and their matched non-tumor adjacent tissues, miR-592 was found to be the most upregulated miRNA in CRC tissues relative to the non-tumor tissue (Table 3), which is consistent with previous miRNA microarray analysis from other groups.[31,32] These might suggest that miR-592 was an oncomir and upregulated in CRC. In order to further validate a correlation of the expression of miR-592 and clinicopathologic stages of CRC, the relative expression of miR-592 in CRC tumor tissues and cell lines was evaluated by a qRT-PCR assay (Figure 1 and Table 1). In line with the previous reports from other groups,[31,32] results of this study also displayed a significant more abundant miR-592 transcript in tumor tissues relative to the matched adjacent non-tumor tissues (Figure 1(a) and Table 1), which is correlated with the TNM stages and lymph node metastasis of CRC (Table 4). In addition, all examined CRC cell lines, including HCT116, LOVE, LS174T, and SW480, also showed an upregulated expression of miR-592 in comparison with normal colon epithelial cell line CCD-18C. (Figure 1(b)). Particularly, LOVO and LS174T cells showed the most abundant miR-592 transcripts among the examined cell lines; therefore, they were chosen as cell models for further investigation in this study.

4.2. The expression of FoxO3A is suppressed in human CRC

In order to interrogate the biological significances of miR-592 in the tumorigenesis of CRC, the online computational miRNA target prediction tool, TargetScan (http://www.targetscan.org), was used to identify potential targets of miR-592 (Supplemental Figure 1). The FoxO3A gene was screened as a potential target of miR-592 for further investigation, partially owing to a conserved seed sequence of this miRNA was possessed within the 3′UTR of its mRNA (Supplemental Figure 1), and its crucial role in pathogenesis of many types of cancers, including the CRC, in which FoxO3A expression was correlated with a favorable

---

Table 3. Top 10 upregulated microRNAs in CRC tissues determined by a microRNA microarray assay.

| MicroRNA   | MirBase number | Fold changes |
|------------|----------------|--------------|
| hsa-miR-592| MIMAT0003260   | 7.524847     |
| hsa-miR-18b| MIMAT0001412   | 5.668322     |
| hsa-miR-18a| MIMAT0000072   | 5.660198     |
| hsa-miR-552| MIMAT0003215   | 5.152094     |
| hsa-miR-96 | MIMAT0000095   | 3.828044     |
| hsa-miR-17 | MIMAT0000700   | 2.922269     |
| hsa-miR-130b| MIMAT0006911  | 2.823839     |
| hsa-miR-21 | MIMAT000076    | 2.324489     |
| hsa-miR-29 | MIMAT000100    | 2.259130     |
| hsa-miR-20b| MIMAT0001413   | 2.113251     |

*Compared with the matched non-tumor adjacent tissues.

---

Figure 1. Aberrant miR-592 transcripts in colorectal cancer tissues and cell lines. (a) Relative expression of miR-592 in CRC tissues. miR-592 expression is significantly upregulated in CRC tissues as compared with the matched adjacent non-tumor tissues ($p < 0.001$). (b) Relative expression of miR-592 in CRC cell lines. miR-592 expression is elevated in CRC cell lines HCT116, LOVO, LS174T and SW480, as compared with the CCD-18C normal intestinal epithelial cell line ($p < 0.001$). Data expressed the mean ± SD; ***$p < 0.001$. 

---
Table 4. Correlation of the demographics and expression of miR-592 in patients with CRC. Data were presented as mean ± SD.

| Demographics               | Number of subjects | p value |
|----------------------------|--------------------|---------|
| Genders                    |                    |         |
| Male                       | 34                 | 0.752   |
| Female                     | 16                 |         |
| Ages ≤62                   | 26                 | 0.506   |
| Ages >62                   | 24                 |         |
| Degrees of differentiation |                    |         |
| High                       | 14                 | 0.474   |
| Medium                     | 22                 |         |
| Low                        | 14                 |         |
| TNM stages                 |                    |         |
| I                          | 5                  | 0.019*  |
| II                         | 26                 |         |
| III                        | 15                 |         |
| IV                         | 4                  |         |
| Lymph node metastasis      |                    |         |
| Yes                        | 22                 | 0.025*  |
| No                         | 28                 |         |

Compared to respective group(s), *p < 0.05.

4.4. An under-expression of miR-592 decreases the capacity of CRC cell migration and proliferation

Since miR-592 was upregulated in CRC cells, in order to explore its oncogenic role in CRC, lentiviral vectors expressing miR-592 inhibitor and a control scramble miRNA, LV-miR-592-inh, and LV-NC were produced, respectively. These viruses were used to infect LOVO and LS174T cells, or generate cell lines stably expressing miR-592 inhibitor or scramble miRNA (Figure 4(a—d)). A significantly reduced miR-592 transcript was observed in cells infected with LV-miR-592-inh, as compared with those transduced with LV-NC vectors as determined by a qRT-PCR assay (Figure 4(e)). Intriguingly, the migration capacity in both miR-592-transduced LOVO and LS174T cells was significantly reduced in comparison with those infected with LV-NC virus, as determined using a scratch assay (p < 0.01) (Figure 5(a—b)). Consistently, the proliferation of CRC cells was also dramatically inhibited when the cells were enforced expression of miR-592 inhibitor, relative to the cell transduced with NC scramble miRNA, as determined by an MTT method (p < 0.01) (Figure 5(c)).

4.5. An under-expression of miR-592 decreases the clonogenicity and tumorigenicity of CRC cells

To better characterize the functionality of miR-592 in CRC tumorigenesis in vitro and in vivo, LOVO and LS174T cells expressing miR-592 inhibitor was employed for evaluating their capacities of clonogenicity in vitro (Figure 6) and xenograft tumorigenesis in vivo (Figure 7). Importantly, an inhibition of miR-592 expression in CRC cells by introducing miR-592 inhibitor exhibited a potency to significantly decrease the capacity of clonogenesis (Figure 6) and tumorigenesis (Figure 7) in both of LOVO and LS174T cells, as compared with those cells infected with LV-NC (p < 0.01). These results imply that miR-592 may play a pivotal role in governing the stemness of colorectal cancer stem cells (CSCs) through a mechanism targeting FoxO3A, suggesting that miR-592 may be a novel target for CRC treatment.

5. Discussion

Increasing evidence has suggested that miRNAs play crucial roles in the development and progression of human CRC. In the present study, we identified miR-592 was a potential oncogene in CRC, which was significantly upregulated in CRC and correlated with the TNM stages and lymph node metastasis of this type of cancer. Importantly, enforced expression of miR-592 inhibitor, antagonimIR-592 inhibitor showed an ability to inhibit the proliferation, migration, and clonogenicity of CRC cells in vitro and to repress tumor growth in a xenograft tumor model of SCID mice. Mechanistically, the oncogenic role of miR-592 is at least in part by directly targeting transcriptional factor FoxO3A.

FoxO3A has been demonstrated as a key transcriptional factor and to be able to regulate signaling networks.[10] In this regard, FoxO3A has an impact on cell proliferation and differentiation and thus were involved in the cell proliferation,
apoptosis, tumorigenesis, and malignant transformation. FoxO3A has been shown to downregulate the well-characterized oncomir miR-21 in part through mechanisms of regulating AP-1 and PTEN pathways in CRC.\[36,37]\] Therefore, it can function as a tumor suppressor that plays a crucial role in the initiation and progression of many cancers, including CRC.\[14,17,20,29,36,38–40]\] Several lines of evidence have revealed that the expression of FoxO3A was significantly reduced in human CRC cell lines and cancer tissues, including the primary and metastatic CRC, and even the metaplastic colorectal tissues, in which the FoxO3A expression was inversely associated with the progression of this cancer type.\[17,20,21,30,38,39,41]\] In CRC, FoxC2 was aberrantly expressed, which promoted CRC cell proliferation through a mechanism of the suppression of FoxO3A and the activation of MAPK and AKT signaling pathways.\[42]\] In this context, FoxO3A was a downstream of AKT signaling.\[35]\] Sarkar et al.\[35]\] recently found that DEAD box protein p68 was overexpressed in colon cancer, which could upregulate AKT expression and consequentially excluded and degraded the nuclear FoxO3A. The Wnt/β-catenin signaling is hyperactivated in CRC, an early study by Tenbaum et al.\[43]\] could subvert FoxO3A to promote the metastasis in colon

---

**Figure 2.** Immunohistochemistry (IHC) staining determined FoxO3A expression in human CRC tumors and matched adjacent tissues. (a–f) Representative images of FoxO3A protein expression determined by IHC staining. (a–c) Images represented the FoxO3A expression in non-CRC tumor adjacent tissues at different magnifications; (d–f) Images represented the FoxO3A expression in CRC tumor tissues at different magnifications. (g) Immunoblotting assay determined FoxO3A protein in CRC tissues and non-tumor intestinal tissues (T = CRC tumor tissue; N = non-tumor intestinal tissue). (h) Semi-quantitative analysis of FoxO3A protein expression using integrated absorbance (IA) in human CRC tissues and the matched adjacent non-tumor tissues. Value was expressed as the average values from each individual sample of CRC tumor tissues or its matched adjacent tissue. The total average value of IA in the CRC tumor tissues was significantly less abundant as compared with the matched adjacent tissues (p < 0.0001, n = 20). Data was expressed as mean ± SD for 20 sets of samples. Bar in A, D: 200 μm; in B, E, F: 100 μm; in C: 50 μm.
cancer. These studies clearly suggest that FoxO3A is a tumor suppressor in CRC. Furthermore, a silencing expression of FoxO3A in CRC cells by small interfering RNA arrested cells at the G2-M phase of cell cycle.\cite{20} In agreement with these findings, the IHC result in the present study also showed a downregulated FoxO3A protein expression in human CRC tissues and cells, which was correlated with a pronounced expression of miR-592 transcript and the progression of the CRC.

Indeed, mounting evidence has recently revealed an upregulated miR-592 transcript in CRC, which plays an oncogenic role and promotes cancer progression and metastasis.\cite{31,32} By microarray analysis of lung adenocarcinoma and lung tissues with CRC metastasis, Kim et al. found that the level of expression of miR-592 in lung with CRC metastasis was sixfolds higher relative to primary lung cancer, suggesting that miR-592 might be a biomarker for diagnosing a primary lung adenocarcinoma and CRC metastasis in lung.\cite{31} Similarly, Liu et al. recently demonstrated that an upregulated miR-592 transcript could be determined in CRC tissues and cell lines, as well as sera of CRC patients, which was significantly correlated with the tumor size, TNM stages, distant metastasis, preoperative CEA level, and a poor overall survival rate in CRC patients. Moreover, by introducing miR-592 mimics or inhibitors into the corresponding CRC cells, the authors further demonstrated that miR-592 could promote cell proliferation, invasion but not

---

Figure 3. Validation of FoxO3A mRNA as a target of miR-592. (a) Sequence of potential binding site of miR-592 in the 3'UTR of human FoxO3A mRNA (top sequence), mutations were introduced into the binding site for generation of mutated FoxO3A 3'UTR (bottom sequence). (b) Validation of miR-592 target using FoxO3A 3'UTR luciferase reporter. C: The expression of FoxO3A was detected by immunoblotting in LS174T cells infected with indicated lentiviral vectors at a week post-infection. The immunoblotting assay showed an increased abundance of FoxO3A protein in the cells infected with the virus expression miR-592 inhibitor (top panel), which was also semi-quantified by a densitometric method (bottom panel). **: Compared with pSicoR/NC group, \( p < 0.01 \). Results represented the mean ± SD from three independent triplicated experiments (\( n = 9 \)).

Figure 4. Generation of CRC cell lines expressing miR-592 inhibitor. Following a DNA cloning strategy, the proviral plasmids were used for production of lentiviral vector LV-miR-592-inh and LV-NC, which express miR-592 inhibitor and scramble control, respectively. These vectors also expressed an EGFP reporter gene for accessing the transduction efficiency. (a–b) The LOVO cells transduced with LV-NC (A) and miR-592-inh (b) showed an infectivity of lentiviral vectors. (c–d) The LS174T cells transduced with LV-NC (c) and miR-592-inh (d) showed an infectivity of lentiviral vectors. (e) qRT-PCR result exhibited a reduced abundance of miR-592 transcript in the LV-miR-592-inh-infected cells relative to the LV-NC-infected cells. Data represents the mean ± SD from three independent experiments. Compared to the LV-NC-infected group, ***: \( p < 0.001 \).
apoptosis in CRC, implying that miR-592 could be severed as a novel and potential biomarker in CRC, and a strategy using anti-miR-592 might be effective treatment for CRC patients.

However, a controversial result was recently reported by another group, the authors stated that miR-592 was downregulated and played a tumor suppressor role, which was able to inhibit cell proliferation and anchorage-independent growth of CRC cells by targeting CCND3 gene expression, as evaluated in eight paired CRC tissues and eight CRC cell lines.

In this study, the authors stated a downregulated miR-592 in CRC tissues and cell lines, in which this miRNA played a tumor suppressor role by targeting CCND3 in CRC. This finding was opposite to the results of ours and two other groups.[31,32] A strikingly upregulated miR-592 was found in CRC tissues with metastasis,[31,32] clearly suggesting that miR-592 might serve as an oncogene in CRC metastasis.

In agreement with their findings, our study further showed that the introduction of miR-592 into CRC cells led to decreased metastatic properties of cell proliferation and migration, clonogenicity, and tumorigenicity. There are two reasons probably that caused the discrepancy among these studies. First, miR-592 could play diverse roles as either an oncomir or a tumor suppressor by targeting distinct mRNAs. Second, miR-592 might dynamically express with the stages of CRC progression. However, the underlying biological roles of miR-592 in CRC still need further investigation.

**Figure 5.** miR-592 promotes CRC cell migration and proliferation in vitro. LOVO and LS174T cells were infected with LV-miR-592-inh or LV-NC, the capability of cell migration was accessed in terms of a scratch assay, and cell proliferative ability was ascertained by an MTT method. (a) Representative images of scratch assays for LOVO cells (top two panels) and LS174T cells (bottom two panels) treated as indicated condition. (b) Relevant quantification of the results of cell migration index. (c) An MTT assay showed an inhibition of cell proliferation in LV-miR-592-inh-infected cells. Compared with LV-NC group, **: p < 0.01. Data in B and C represented the mean ± SD from three independent triplicated experiments (n = 9).

**Figure 6.** miR-592 enhances the stemness of CRC cells determined by a clonogenic assay. LOVO and LS174T cells stably expressing miR-592-inh or miRNA NC were the formation of cell colonies using a clonogenic assay in 35-mm dishes. (a) Representative images of clonogenic assay for LOVO cells (top panel) and LS174T cells (bottom panel). (b) Relevant quantification of the results of cell clonogenic index, which showed an inhibition of clonogenic capacity in LV-miR-592-inh-infected cells. Compared with LV-NC group, **: p < 0.01. Data in B represented the mean ± SD from three independent triplicated experiments (n = 9).
Nevertheless, given the fact that FoxO3A is a tumor suppressor, an under-expression of FoxO3A will exhibit an enhanced metastatic properties and tumorigenicity in many cancer types, including CRC.[17, 20, 21, 30, 38, 39, 41] Consistent with the finding in this study, an upregulated miR-592 was previously observed in prostate cancer cells, in which it promoted cancer cell proliferation by targeting FoxO3A.[46] In addition to miR-592, other miRNAs that could target FoxO3A were also identified in CRC, such as miR-153,[47] miR-224,[48] and miR-96.[21] For instance, miR-96 has also demonstrated a capacity to directly or indirectly target FoxO3A in CRC and other cancers. miR-96 was reported to be overexpressed in human CRC and was able to enhance tumor cell viability, colony formation ability, and cell cycle progression by directly targeting p53 inducible nuclear protein 1, FOXO1, and FoxO3A.[21] Such an ability of miR-96 to target FoxO3A was also demonstrated in breast cancer.[15] Of note, the biological roles of miR-592 in tumorigenesis are cancer type-dependent, that is miR-592 showed both oncogenic and tumor suppressor roles in distinct cancer types. For example, it was downregulated in hepatocellular carcinoma and served as a tumor suppressor. miR-592 was able to suppress cell growth in hepatocellular carcinoma cell line HepG2 by targeting DEK oncogene.[45]

Accumulating evidence has demonstrated a potential effectiveness for suppressing tumor growth and reversing drug resistance by targeting oncomirs using antisense miRNAs. In this regard, miR-23a and miR-21 have spurred the most interest, owing to their aberrantly expressed in CRC cells.[49] For instance, inhibition of miR-21 by antisense miR-21 (antagomiR-21) promoted the differentiation of CRC HCT-116 or HT-29 cells, which was accompanied by significant decreases of the expression of CRSC marker CD44, capacity of colonsphere formation, and Wnt signaling activity but an increase of PDCD4 expression.[50] Similarly, miR-23a was found to be elevated in 5-FU CRC cells, and its target APAF-1 along with caspase-3 and -7 was downregulated in these cells. Interestingly, introduction of miR-23a antisense into 5-FU resistant cells showed an increased level of APAF-1, along with an enhanced activation of caspase-3 and -7, which subsequently enhanced the 5-FU induced apoptosis in these cells.[51] By employing a similar strategy, Ge et al. investigated the effect of antagomiR-27a in glioblastoma.[22] As aberrant miR-21 and miR-592 seen in CRC, more abundant miR-27a transcript was detected in specimens from glioblastoma comparing with normal human brain tissues. More interestingly, FoxO3A mRNA was identified as a novel target of miR-27a, transduction of antagomiR-27a (miR-27a inhibitor) showed an ability to significantly inhibit the invasion and proliferation of U87 glioblastoma cells and reduce the growth of glioblastoma xenograft in SCID mice.[22] Consistent with this finding, our data also showed that an introduction of miR-592 inhibitor led to the suppression of CRC cell proliferation and migration, and a decreased capacity of CRC cell clonogenicity and tumorigenicity in vitro and in vivo.

6. Conclusion

Collectively, less abundant FoxO3A protein was detected in CRC tissues and cell lines, accompanied with the striking upregulation of miR-592. An under-expression of miR-592 exhibited an ability to promote cell apoptosis, reduce metastatic potentials and the capacity of clonogenicity in vitro, as well as decrease the ability of tumorigenicity of CRC cells in vivo, through a mechanism by which miR-592 directly targeted FoxO3A. We thus identified miR-592 as an oncogene in CRC, which warranted for further investigation as a novel target for prognosis, prevention, and the treatment of this disease.
Declarations of interest

This work was supported by a grant from Natural Science Foundation of China (No: 81263009). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

References

Papers of special note have been highlighted as:
- of considerable interest
- of interest

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin. 2015;65:5–29.
2. Garofalo M, Croce CM. MicroRNAs as therapeutic targets in chemoresistance. Drug Resist Update. 2013;16:47–59.
3. Cojoc M, Mabert K, Maders MH, et al. A role for cancer stem cells in therapy resistance: cellular and molecular mechanisms. Semin Cancer Biol. 2015;31:16–27.
4. Kartal-Yandim M, Adan-Gokbulut A, Baran Y. Molecular mechanisms of drug resistance and its reversal in cancer. Crit Rev Biotechnol. 2015;31:1–11. [Epub ahead of print]
5. Lippert TH, Ruoff HJ, Yolm M. Intrinsic and acquired drug resistance in malignant tumors. The main reason for therapeutic failure. Arzneimittelforschung. 2008;58:261–264.
6. Wang J, Du Y, Liu X, et al. MicroRNAs as regulator of signaling networks in metastatic colon cancer. Biomed Res Int. 2015;2015:823620.
7. Cho WC. Oncomirs: the discovery and progress of microRNAs in cancers. Mol Cancer. 2007;6:60.
8. Di Leva G, Garofalo M, Croce CM. MicroRNAs in cancer. Annu Rev Pathol. 2014;9:287–314.
9. Wang J, Liu S, Yin Y, et al. FOXO3-mediated up-regulation of Bim contributes to rhein-induced cancer cell apoptosis. Apoptosis. 2015;20:399–409.
10. Nho RS, Hergett P, FoxO3a and disease progression. World J Biol Chem. 2014;5:346–354.
11. Cheng CW, Chen PM, Hsieh YH, et al. Foxo3a-mediated upexpression of microRNA-622 suppresses tumor metastasis by repressing hypoxia-inducible factor-1alpha in erk-resistant of lung cancer. Oncotarget. 2015;6:44222–44238.
12. Finnberg N, El-Deiry WS. Activating FOXO3a, NF-kappaB and p53 as using targeting IKKs: an effective multi-faceted targeting of the tumor cell phenotype? Cancer Biol Ther. 2004;3:614–616.
13. Kong W, He L, Coppola M, et al. MicroRNA-155 regulates cell survival, growth, and chemosensitivity by targeting FOXO3a in breast cancer. J Biol Chem. 2010;285:17869–17879.
14. Levanon K, Sapoznik S, Bahar-Shany K, et al. FOXO3a loss is a frequent early event in high-grade pelvic serous carcinogenesis. Oncogene. 2014;33:4424–4432.
15. Lin H, Dai T, Xiong H, et al. Unregulated miR-96 induces cell proliferation in human breast cancer by downregulating transcriptional factor FOXO3a. PLoS one. 2010;5:e15797.
16. Liu H, Yin J, Wang H, et al. FOXO3a modulates WNT/β-catenin signaling and suppresses epithelial-to-mesenchymal transition in prostate cancer cells. Cell Signal. 2015;27:510–518.
17. Yan Y, Lackner MR. FOXO3a and β-catenin co-localization: double trouble in colon cancer? Nat Med. 2012;18:854–856.
18. Zhao F, Lam EW. Role of the forkhead transcription factor FOXO-FOXMI axis in cancer and drug resistance. Front Med. 2012;637:380.
19. Carbajo-Pescador S, Mauriz JL, Garcia-Palomo A, et al. FoxO proteins: regulation and molecular targets in liver cancer. Curr Med Chem. 2014;21:1231–1246.
20. He LY, Wei X, Du L, et al. Remarkably reduced expression of FoxO3a in metastatic colorectum, primary colorectal cancer and liver metastasis. J Huazhong Univ Sci Technol Med Sci. 2013;32:205–211.
21. Gao F, Wang W. MicroRNA-96 promotes the proliferation of colorectal cancer cells and targets tumor protein p53 inducible nuclear protein 1, forkhead box protein O1 (FOXO1) and FOXO3a. Mol Med Rep. 2015;11:1200–1206.
22. Providing the evidence the FOXO3A is a target of oncomir miR-96 in CRC.
23. Ge YF, Sun J, Jin CJ, et al. AntagomiR-27a targets FOXO3A in glioblastoma and suppresses U87 cell growth in vitro and in vivo. Asian Pac J Cancer Prev. 2013;14:963–968.
24. Ji WG, Zhang XD, Sun XD, et al. miRNA-155 modulates the malignant biological characteristics of NK/T-cell lymphoma cells by targeting FOXO3a gene. J Huazhong Univ Sci Technol Med Sci. 2014;34:882–888.
25. Li X, Du N, Zhang Q, et al. MicroRNA-30d regulates cardiomyocyte pyroptosis by directly targeting foxo3a in diabetic cardiomyopathy. Cell Death Dis. 2014;5:e1479.
26. Ling N, Gu J, Lei Z, et al. microRNA-155 regulates cell proliferation and invasion by targeting FOXO3A in glioma. Oncol Rep. 2013;30:2111–2118.
27. Providing the evidence the FOXO3A is a target of oncomir miR-155.
28. Nho RS, Im J, Ho YY, et al. MicroRNA-96 inhibits FoxO3a function in IPF fibroblasts on type I collagen matrix. Am J Physiol Lung Cell Mol Physiol. 2014;307:L632–L342.
29. Chi H. miR-194 regulated AGK and inhibited cell proliferation of oral squamous cell carcinoma by reducing PI3K-Akt-FoxO3a signaling. Biomed Pharmacother. 2015;71:53–57.
30. Chiacchiera F, Simone C. Inhibition of p38alpha unveils an AMPK-FoxO3A axis linking autophagy to cancer-specific metabolism. Autophagy. 2009;5:1030–1033.
31. Liu X, Feng J, Tang L, et al. The regulation and function of miR-21-FOXO3A-miR-34b/c signaling in breast cancer. Int J Mol Sci. 2015;16:3148–3162.
32. Myant K, Sansom OJ. More, more, more: downregulation of a MK5-FoxO3a-miR-34b/c pathway further increases c-Myc levels in colorectal cancer. Mol Cell. 2011;41:369–370.
33. Kim J, Lim NJ, Jang SG, et al. miR-592 and miR-552 can distinguish between primary lung adenocarcinoma and colorectal cancer metastases in the lung. Anticancer Res. 2014;34:2297–2302.
34. First report providing the evidence of miR-592 as a biomarker for CRC metastasis.
35. Liu M, Zhi Q, Wang W, et al. Up-regulation of miR-592 correlates with tumor progression and poor prognosis in patients with colorectal cancer. Biomed Pharmacother. 2015;69:214–220.
36. A strong evidence of miR-592 as a biomarker for CRC metastasis in clinical settings.
37. Li H, Li C, Dai R, et al. Expression of acetylated histone 3 in the spinal cord and the effect of morphine on inflammatory pain in rats. Neural Regen Res. 2012;7:517–522.
38. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001;29:e45.
39. Sarkar M, Khare V, Guturi KK, et al. The DEAD box protein p68: a crucial regulator of AKT/FOXO3a signaling axis in oncogenesis. Oncogene. 2015;34:5843–5856.
40. Fluckiger A, Dumont A, Derangere V, et al. Inhibition of colon cancer growth by docosahexaenoic acid involves autocrine production of TNFalpha. Oncogene. 2016;DOI:10.1038/onc.2015.523. [Epub ahead of print].
41. Saxena A, Shob M, Ramana KV, et al. Aldose reductase inhibition suppresses colon cancer cell viability by modulating microRNA-21 mediated programmed cell death 4 (PDCD4) expression. Eur J Cancer. 2013;49:3311–3319.
42. Germanni A, Matrone A, Grossi V, et al. Targeted therapy against chemoresistant colorectal cancers: Inhibition of p38alpha modulates the effect of cisplatin in vitro and in vivo through the tumor suppressor FoxO3A. Cancer Lett. 2014;344:110–118.
43. Luo H, Yang Y, Duan J, et al. PTEN-regulated AKT/FoxO3a/Bim signaling contributes to reactive oxygen species-mediated apoptosis in selenite-treated colorectal cancer cells. Cell Death Dis. 2013;4:e481.
44. Taylor S, Lam M, Pararasa C, et al. Evaluating the evidence for targeting FOXO3A in breast cancer: a systematic review. Cancer Cell Int. 2015;15:1.
41. Chiacciola F, Simone C. The AMPK-FoxO3A axis as a target for cancer treatment. Cell Cycle. 2010;9:1091–1096.
42. Cui YM, Jiang D, Zhang SH, et al. FOXC2 promotes colorectal cancer proliferation through inhibition of FOXO3a and activation of MAPK and AKT signaling pathways. Cancer Lett. 2014;353:87–94.
43. Tenbaum SP, Ordonez-Moran P, Puig I, et al. beta-catenin confers resistance to PI3K and AKT inhibitors and subverts FOXO3a to promote metastasis in colon cancer. Nat Med. 2012;18:892–901.
44. Liu Z, Wu R, Li G, et al. MiR-592 inhibited cell proliferation of human colorectal cancer cells by suppressing of CCND3 expression. Int J Clin Exp Med. 2015;8:3490–3497.

- A report demonstrated the tumor suppressor role of miR-592 in CRC.
45. Li X, Zhang W, Zhou L, et al. MicroRNA-592 targets DEK oncogene and suppresses cell growth in the hepatocellular carcinoma cell line HepG2. Int J Clin Exp Pathol. 2015;8:12455–12463.
46. Lv Z, Rao P, Li W. MiR-592 represses FOXO3 expression and promotes the proliferation of prostate cancer cells. Int J Clin Exp Med. 2015;8:15246–15253.
47. Zhang L, Pickard K, Jenei V, et al. miR-153 supports colorectal cancer progression via pleiotropic effects that enhance invasion and chemotherapeutic resistance. Cancer Res. 2013;73:6435–6447.
48. Liao WT, Li TT, Wang ZG, et al. microRNA-224 promotes cell proliferation and tumor growth in human colorectal cancer by repressing PHLPP1 and PHLPP2. Clin Cancer Res. 2013;19:4662–4672.
49. Liu X, Fu Q, Du Y, et al. MicroRNA as regulators of cancer stem cells and chemoresistance in colorectal cancer. Curr Cancer Drug Targets. 2015;in press.
50. Yu Y, Sarkar FH, Majumdar AP. Down-regulation of miR-21 induces differentiation of chemoresistant colon cancer cells and enhances susceptibility to therapeutic regimens. Transl Oncol. 2013;6:180–186.

- Providing the evidence of the potential of antagomir in cancer treatment.
51. Shang J, Yang F, Wang Y, et al. MicroRNA-23a antisense enhances 5-fluorouracil chemosensitivity through APAF-1/caspase-9 apoptotic pathway in colorectal cancer cells. J Cell Biochem. 2014;115:772–784.