

## MicroRNA-95 Promotes Cell Proliferation and Targets Sorting Nexin 1 in Human Colorectal Carcinoma

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### Abstract

MicroRNAs (miRNAs) are strongly implicated in cancer but their specific roles and functions in the major cancers have yet to be fully elucidated. In this study, we defined the oncogenic significance and function of miR-95, which we found to be elevated in colorectal cancer (CRC) tissues by microarray analysis. Evaluation of an expanded CRC cohort revealed that miR-95 expression was up-regulated in nearly half of the tumors examined (42/87) compared with the corresponding noncancerous tissues. Ectopic overexpression of miR-95 in human CRC cell lines promoted cell growth *in vitro* and tumorigenicity *in vivo*, whereas RNAi-mediated silencing of miR-95 decreased cell growth ratio. Mechanistic studies revealed that miR-95 repressed the expression of reporter gene coupled to the 3'-untranslated region of sorting nexin 1 (SNX1), whereas miR-95 silencing up-regulated SNX1 expression. Moreover, miR-95 expression levels correlated inversely with SNX1 protein levels in human CRC tissues. RNAi-mediated knockdown of SNX1 phenocopied the proliferation-promoting effect of miR-95, whereas overexpression of SNX1 blocked miR-95-induced proliferation of CRC cells. Taken together, these results demonstrated that miR-95 increases proliferation by directly targeting SNX1, defining miR-95 as a new oncogenic miRNA in CRC. *Cancer Res*; 71(7); 2582–9. ©2011 AACR.

### Introduction

MicroRNAs (miRNAs) are a class of approximately 22 nucleotide noncoding RNA molecules that negatively regulate the expression of a wide variety of genes mainly through direct interaction with the 3'-untranslated regions (3'UTR) of their corresponding mRNA targets (1). It has been estimated that miRNAs regulate up to one third of the total human genes at the posttranscriptional level (2), indicating that miRNAs have pivotal roles in physiological and pathological processes. Increasing evidence shows that the deregulation of miRNAs is involved in a wide range of diseases, including human cancers. These data highlight the important roles of miRNAs

in tumor development and provide new insights into the molecular mechanisms underlying carcinogenesis (3, 4). The number of verified miRNAs is increasing rapidly, and the latest version of miRBase (release 16.0, September 2010, <http://www.mirbase.org/>) has annotated over 1,000 miRNA sequences in the human genome. However, the roles of most of these miRNAs in the physiological and pathological processes remain to be elucidated.

Colorectal carcinoma (CRC) is the third most common cancer worldwide. Despite achievements in the treatment in the few past decades, CRC remains a major public health concern. The molecular pathogenesis of CRC is complicated and poorly understood. Although previous studies found that many protein-coding genes, including *p53*, *APC*, *K-ras*, and *DCC*, are involved in the development and progression of CRC, the roles and potential mechanisms of miRNAs in CRC are still largely unknown. Only few miRNAs, such as let-7 (5), miR-21 (6–8), miR-143 (9, 10), miR-145 (11), miR-135 (12), and 196a (13), have been studied for their roles in colorectal carcinogenesis.

In this study, we investigated the miRNA expression profiles in CRC and identified a new proliferation-promoting miRNA, miR-95, which is frequently up-regulated in CRC. Moreover, we identified sorting nexin 1 (SNX1), a putative tumor suppressor in CRC (14), as the direct functional target of miR-95.

### Materials and Methods

#### Human tissues and cell lines

A total of 146 pairs of human primary CRC and adjacent noncancerous tissues (NCT) were collected between 2004 and

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2007 at Fudan University Shanghai Cancer Center. Tissue samples were immediately snap-frozen in liquid nitrogen. Both tumor and adjacent NCT were histologically examined. All human materials were obtained with informed consent, and this project was approved by the Clinical Research Ethics Committee of Fudan University Shanghai Cancer Center. Clinical information of these patients is listed in Supplementary Table S1.

Five human CRC cell lines, including HCT-116, HCT-8, LoVo, SW480, and Colo-205, were purchased from American Type Culture Collection (ATCC). HCT-8 and Colo-205 were maintained in RPMI 1640, HCT-116 in McCoy's 5a, LoVo in F12-K, and SW480 in Leibovitz's L-15 medium. Media were supplemented with 10% fetal bovine serum. The cells were incubated under the conditions recommended by ATCC.

### Microarray analysis

Total RNA was extracted from 10 tumor tissues and paired NCTs using TRIzol reagent (Invitrogen). RNA concentration was assessed with a NanoDrop spectrophotometer (NanoDrop Technologies) and RNA integrity was verified using an Agilent 2100 bioanalyzer (Agilent). MiRNA microarray profiling was performed as previously described (15). Data analysis was performed by using GeneSpring GX software (Agilent). A miRNA was designated as overexpressed if expression in tumor tissues was >2.0-fold that in normal colorectal epithelial tissues.

### RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted using TRIzol reagent. cDNA was synthesized with the PrimeScript RT reagent Kit (TaKaRa). Quantitative RT-PCR (qRT-PCR) analyses were carried out to detect mRNA expression using SYBR Premix Ex Taq (TaKaRa), and  $\beta$ -actin was used as an internal control. TaqMan microRNA assays (Applied Biosystems) were used to quantitate the expression levels of mature miR-95 according to the provided protocol, and U6 small nuclear RNA was used as an internal control.

### Vector constructs

The human pri-miR-95 sequence was amplified from normal human genomic DNA and cloned into pMD-18T vector (Takara) and then subcloned (BamH I and EcoR I) into the lentivirus expression vector pWPXL (a generous gift from Dr. T. Didier) to generate pWPXL-miR-95.

The 3'UTR of potential target genes of miR-95 were amplified and cloned into the region directly downstream of a CMV promoter-driven firefly luciferase cassette in a pcDNA3.0 vector (p-Luc). The mutant 3'UTR of *SNX1*, which carried the mutated sequence in the complementary site for the seed region of miR-95, was generated based on the p-Luc-SNX1 3'UTR-WT plasmid by overlap-extension PCR. The open reading frame (ORF) of *SNX1* was amplified and cloned into pWPXL vector (a generous gift from Professor Didier TRONO). The primers used are displayed in Supplementary Table S2.

### Lentivirus production and transduction

Virus particles were harvested 48 hours after pWPXL-miR-95 (or pWPXL-SNX1) cotransfection with the packaging

plasmid psPAX2, and VSV-G envelope plasmid, pMD2.G (a generous gift from Professor Didier TRONO), into HEK 293T cells using LipoFectamine 2000 reagent (Invitrogen). HCT-116 and LoVo cells, which express lower levels of miR-95 than other CRC cell lines (Supplementary Fig. S1A), were infected with recombinant lentivirus-transducing units plus 6  $\mu$ g/mL polybrene (Sigma).

### Oligonucleotide transfection

MiR-95 mimics was synthesized by Genepharma, Shanghai, China. MiR-95 inhibitor (anti-miR-95, chemically modified antisense oligonucleotides designed to target specifically against mature miR-95), and *SNX1* siRNA (target sequence: CCACGTGATCAAGTACCTT) were synthesized Ribobio, Guangzhou, China. Oligonucleotide transfection was performed with Lipofectamine 2000 reagents (Invitrogen). The final concentration of miR-95 mimics or anti-miR-95 in the transfection system was 50 nM.

### Cell proliferation assay and colony formation assay

A cell proliferation assay was performed with the Cell Counting Kit-8 (CCK-8; Dojindo) according to the manufacturer's instruction. For the colony formation assay, 1,000 cells were placed in each well of 6-well plate and maintained in media containing 10% FBS for 10 days. Colonies were fixed with methanol and stained with 0.1% crystal violet in 20% methanol for 15 minutes. The number of colonies was counted using an inverted microscope.

### Tumor formation assay in a nude mouse model

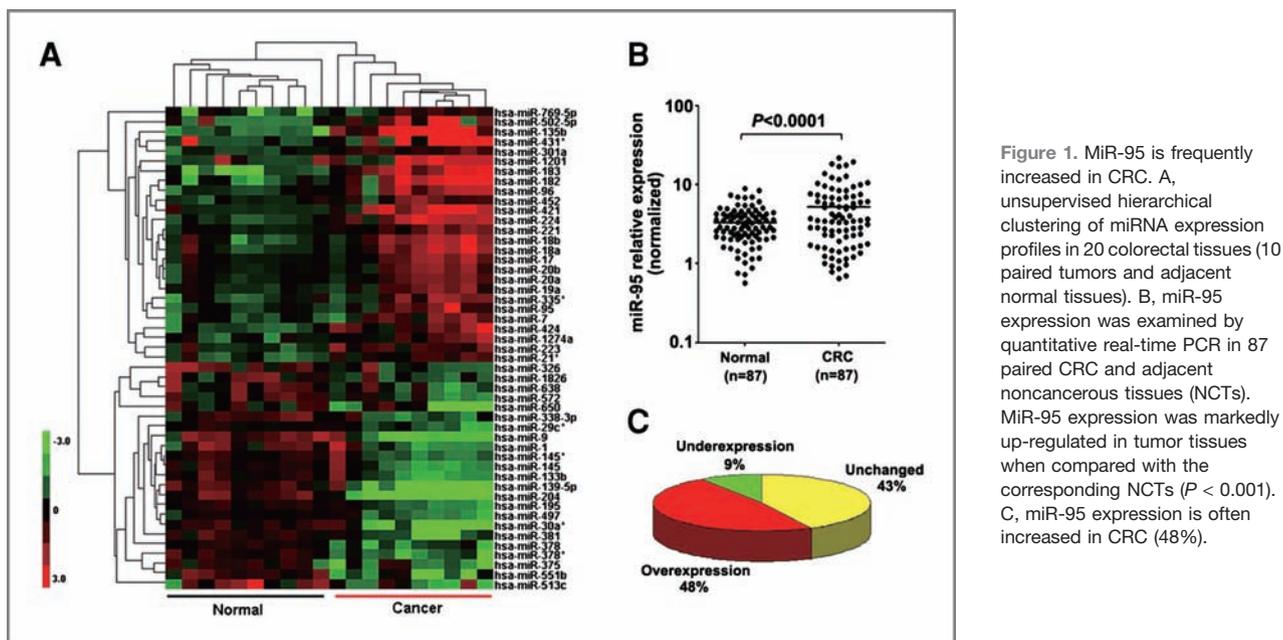
Athymic BALB/c nude mice were maintained under specific pathogen-free conditions in the Department of Experimental Pathology, Shanghai Cancer Institute. Mice were manipulated and housed according to protocols approved by the Shanghai Medical Experimental Animal Care Commission. Tumor cells stably expressing miR-95 (LoVo or HCT-116) or vector control, were harvested from subconfluent cell culture plates, washed with PBS, and resuspended at a concentration of  $2 \times 10^7$  cells per milliliter of PBS. Of the suspending cells, 0.1 mL was subcutaneously injected into either side of the flank of the same female nude mouse at 5 weeks of age. Tumor growth was examined twice a week. After 6 weeks, these mice were sacrificed and examined for the growth of subcutaneous tumors.

### Luciferase assay

HEK 293T cells were cultured in 96-well plates and cotransfected with 200 ng of pWPXL-miR-95 (or pWPXL), 50 ng luciferase reporter, and 10 ng pRL-CMV *Renilla* luciferase reporter using lipofectamine 2000. After 48 hours of transfection, firefly and *Renilla* luciferase activities were measured using the dual-luciferase reporter assay system (Promega).

### Western blot

Proteins were separated on 10% SDS-PAGE gel and then transferred to nitrocellulose membrane (Bio-Rad). After blocked with 5% nonfat milk, the membrane was incubated with mouse anti-SNX1 McAb (1:400, BD Transduction



Laboratories), mouse anti- $\beta$ -actin McAb (1:1000, Sigma), or antibodies against (Tyr 1068) phosphorylated EGFR (1:1000, Abcam) or total EGFR (1:1000, Cell Signaling). The proteins were visualized using ECL reagents (Pierce).

#### Immunohistochemical staining

Tissue arrays were constructed using 146 pairs of CRC tissues and adjacent NCTs. Immunohistochemical staining was performed on 5- $\mu$ m sections of paraffin-embedded human CRC tissues and matched NCTs to determine the expression of SNX1. Briefly, the slides were incubated in 1  $\mu$ g/mL SNX1 antibody (1:250, BD Transduction Laboratories) overnight. Subsequent steps were performed using Universal Daka LSAB + kit and Peroxidase (LASB + Kit, HRP) according to the manufacturer's instruction (DAKO). The intensity of the SNX1 staining pattern was quantitated as described (14).

#### Statistical analysis

The results were presented as mean  $\pm$  S.E.M. The data were subjected to Student's  $t$  test unless otherwise specified ( $\chi^2$  test). A  $P$  value of less than 0.05 was considered statistically significant.

## Results

### Expression of miR-95 is frequently increased in human CRC tissues

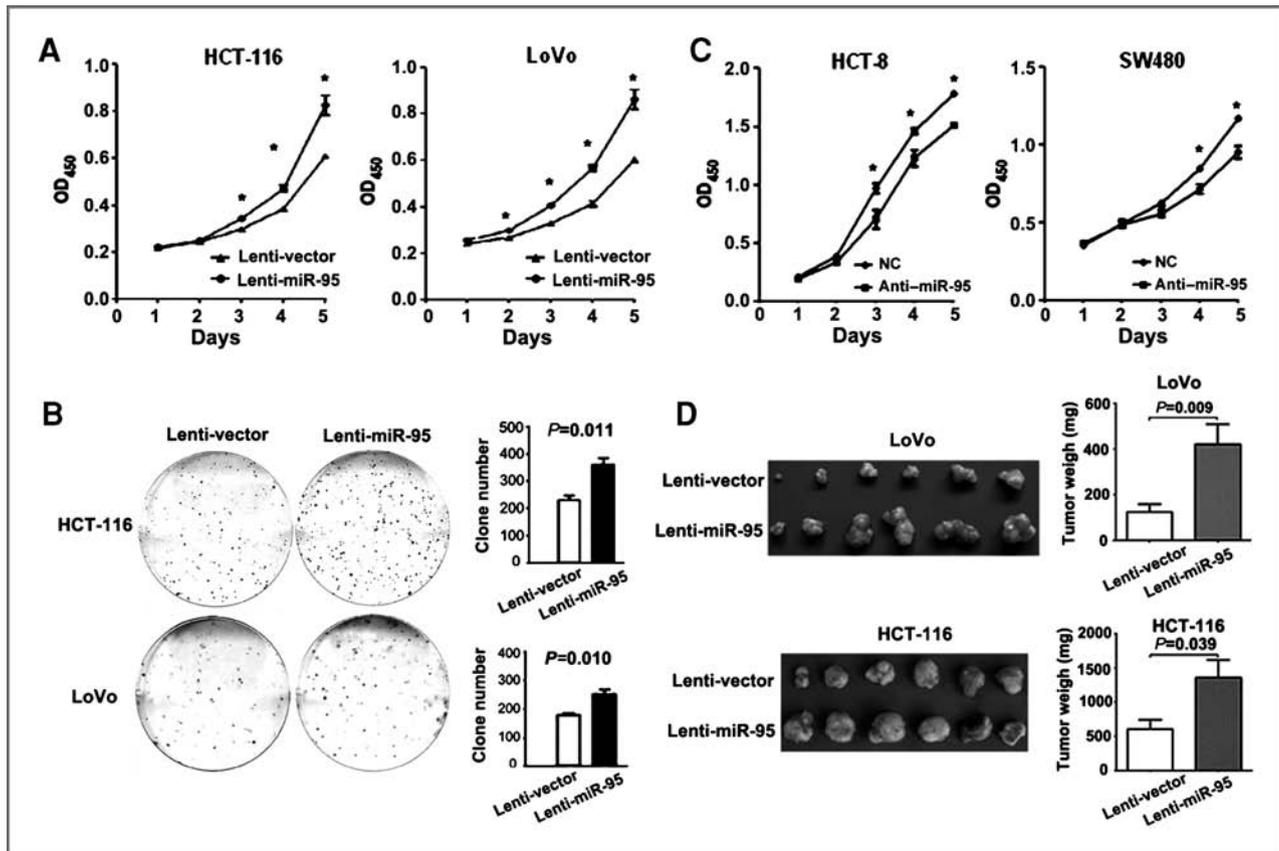
MiRNA expression profiles in 10 paired CRC tissues and NCTs were analyzed using a microarray to screen differentially expressed miRNAs in CRC. Of the 866 human miRNAs analyzed, 49 exhibited significantly differential expression in CRC tissues (Fig. 1A and Supplementary Table S3). Unsupervised hierarchical clustering with these 49 significantly dysregulated miRNAs was able to distinguish the CRCs from corresponding

NCTs (Fig. 1A). To find new potential CRC-related miRNAs from these miRNAs, we selected several targets (miR-31, -95, -223, -139-5p, -30a\*, and -204) to evaluate their influence on the growth of CRC cell using miRNA mimics transfection assay; and found that miR-95 show the strongest proliferation-promoting effect (data not shown). In addition, upregulation of miR-95 has been observed in multiple types of cancer including CRC (16, 17), lung cancer (18), and pancreatic cancer (19, 20), suggesting that deregulation of miR-95 may play an essential role in tumorigenesis.

To further confirm the upregulation of miR-95 in CRC, the expression level of miR-95 was examined in an expanded CRC cohort consisting of 87 pairs of tumor tissues and corresponding NCTs using qRT-PCR assay. Consistent with the microarray data, miR-95 expression was up-regulated in nearly half of the tumors examined (42/87) when compared with the adjacent NCTs ( $P < 0.001$ , Fig. 1B and C). However, no significant association was found between miR-95 expression in CRC and tumor size, location, stage, or differentiation ( $P > 0.05$ ).

### MiR-95 promotes CRC cell proliferation *in vitro* and *in vivo*

To determine the impact of miR-95 on CRC cell proliferation, we established HCT-116 and LoVo transfectants stably expressing miR-95 using lentivirus infection (Supplementary Fig. S1B). The cell proliferation assays and colony formation assays revealed that overexpression of miR-95 can significantly promote CRC cell proliferation ( $P < 0.05$ , Fig. 2A and B), whereas RNAi-mediated silencing of miR-95 decreased cell growth ratio ( $P < 0.05$ , Fig. 2C). Furthermore, overexpression of miR-95 could significantly promote the tumorigenicity of CRC cells in nude mice (Fig. 2D). Collectively, our data indicated that miR-95 exerts a growth-promoting function in human CRC.



**Figure 2.** MiR-95 enhances CRC cell growth *in vitro* and *in vivo*. A, the effect of miR-95 on cell proliferation of miR-95-overexpressing cells (HCT-116 and LoVo). B, the effect of miR-95 on colony formation of CRC cells (HCT-116 and LoVo). Representative results of colony formation of Lenti-vector controls (left), miR-95-transfected (right) HCT-116 and LoVo cells. C, knockdown of miR-95 resulted in decreased growth ratio of the CRC cell lines (HCT-8 and SW480). D, the effect of miR-95 on tumor formation in a nude mouse xenograft model. Lenti-vector- and miR-95-transfected LoVo cells ( $2 \times 10^6$ ) were injected s.c. into either flank of the same nude mouse (left, Lenti-vector; right, miR-95). The tumor weight of the miR-95 group was significantly increased compared with the control group. Two mice with tumor necrosis were excluded from the comparison of tumor weight. \*,  $P < 0.05$ .

### SNX1 is a direct target of miR-95

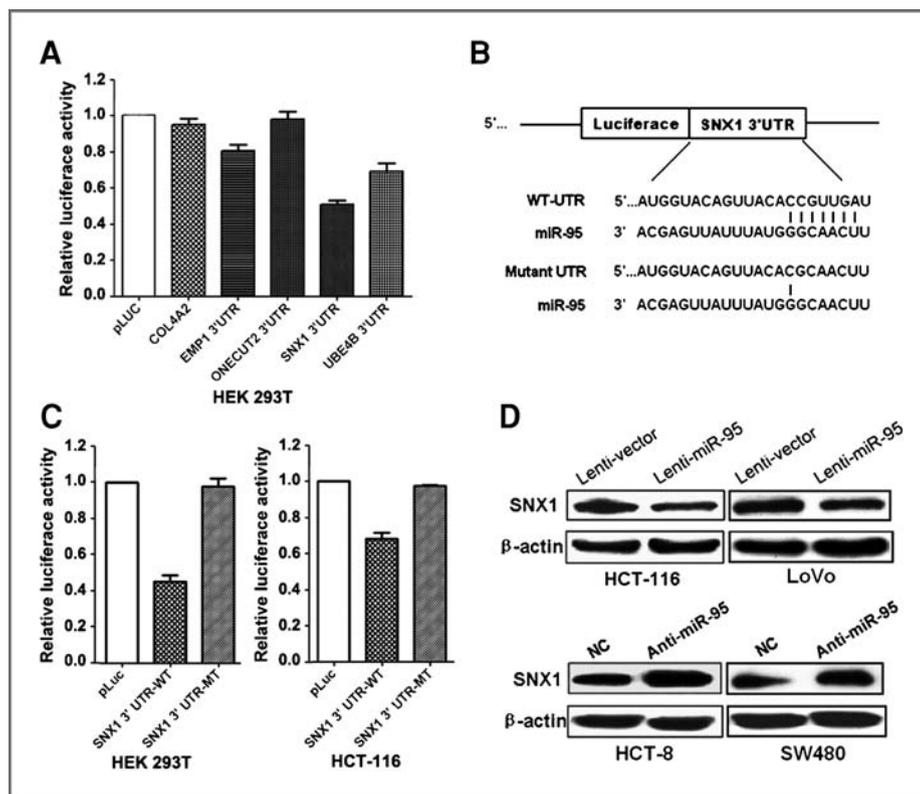
It is generally accepted that miRNAs exert their function by downregulating the expression of their downstream target genes. Thus, miR-95 may execute its growth-promoting function by inhibiting its targets that normally have tumor suppressive effect. Basing on this rationale, 5 potential genes (*COL4A2*, *EMPI*, *ONECUT2*, *SNX1*, and *UBE4B*) were selected using TargetScan 5.1 (21). We cloned the 3'UTRs of these 5 genes into a luciferase reporter vector to evaluate the influence of miR-95 on expression of said reporter gene using a luciferase assay. The results revealed that miR-95 could inhibit the expression of reporter gene in recombinant plasmids of *SNX1*, *UBE4B*, and *EMPI* 3'UTRs, especially *SNX1* 3'UTR (Fig. 3A).

To test whether *SNX1* is a direct target for miR-95, we mutated the predicted binding site of miR-95 on the *SNX1* 3'UTR (Fig. 3B), and found that the mutant *SNX1* 3'UTR was completely refractory to miR-95-mediated luciferase reporter repression (Fig. 3C). In concordance with these results, endogenous *SNX1* protein levels were also downregulated in miR-95-overexpressed cells and could be

restored in miR-95-depleted cells (Fig. 3D). The *SNX1* mRNA levels were not decreased in miR-95 stable cell lines when compared with cells transfected with an empty vector (data not shown).

### Downregulation of *SNX1* inversely correlated with miR-95 expression in CRC

To further evaluate the relationship between miR-95 and *SNX1* in human CRC, we detected the expression of *SNX1* and miR-95 in 146 paired CRC and NCT tissues using IHC and qRT-PCR, respectively. Of the 146 cases, 114 tumors showed decreased *SNX1* expression when compared with paired NCTs (Fig. 4A and B). The expression levels of *SNX1* in tumor tissues inversely correlated with the miR-95 levels (Spearman  $r = -0.197$ ,  $P = 0.017$ ;  $\chi^2 = 5.678$ ,  $P = 0.017$ ; Fig. 4C and Table 1), suggesting that the decreased *SNX1* expression might result from miR-95 overexpression in human CRC. In addition, decreased immunoreactivity of *SNX1* correlated with poor tumor differentiation (Spearman  $r = -0.177$ ,  $P = 0.032$ ). No association was observed between *SNX1* staining and tumor size, grade or lymph node metastasis ( $P > 0.05$ ).



**Figure 3.** MiR-95 suppresses SNX1 expression by directly targeting its 3'UTR. **A**, 3'UTRs of 5 potential miR-95 targets (*COL4A2*, *EMP1*, *ONECUT2*, *SNX1*, and *UBE4B*) were assessed using the luciferase reporter assay. The activities of miR-95 were determined by luciferase reporter assays after transfection with miR-95-expressing plasmids. Luciferase activity was detected 48 hours after transfection. The luciferase activity of each sample was normalized to the *Renilla* luciferase activity. The normalized luciferase activity of p-Luc in each experiment was set as relative luciferase activity 1. **B**, putative miR-95-binding sequence in the *SNX1* 3'UTR. Mutation was generated in the *SNX1* 3'UTR sequence in the complementary site for the seed region of miR-95 as indicated. **C**, analysis of the luciferase activity of the luciferase reporter plasmid containing either wild-type (WT) or mutant (MT) *SNX1* 3'UTR. **D**, suppressed expression of endogenous SNX1 by miR-95. Western blot analysis was used to monitor the expression level of endogenous SNX1 in CRC cells after 48 hours transfection with miR-95 or empty vector (lenti-vector) and in CRC cells transfected with miR-95 inhibitor (anti-miR-95) or control RNA duplex (NC).

### MiR-95 promotes tumor proliferation via directly targeting SNX1 in CRC

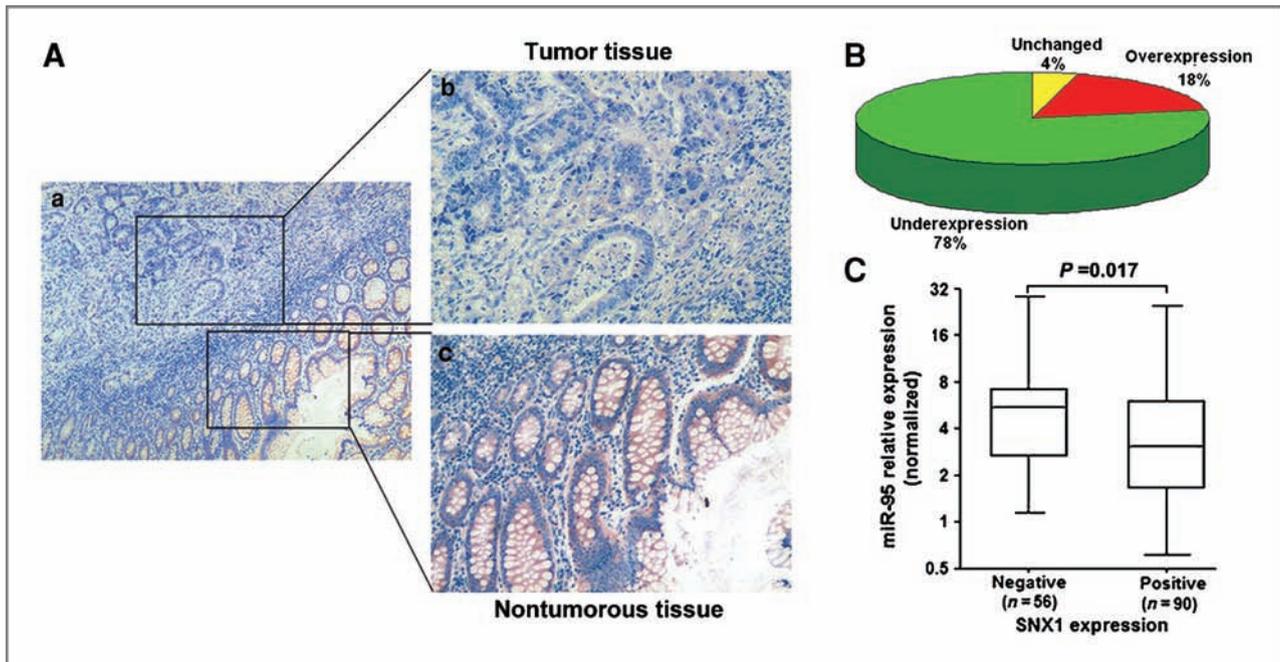
To examine whether SNX1 might function in miR-95-induced CRC cell proliferation, we inhibited the SNX1 expression with siRNA and revealed that SNX1-depleted cells showed increased proliferation, which phenocopied the proliferation-promoting effect of miR-95 (Fig. 5A). Cotransfection experiments using siSNX1 and anti-miR-95 also showed that miR-95 silencing could not repress proliferation in SNX1-depleted HCT-8 cells (Fig. 5B). Furthermore, we revealed that SNX1 overexpression could significantly abrogate miR-95-induced cell growth (Fig. 5C). In addition, overexpression of miR-95 could increase EGFR phosphorylation and inhibition of miR-95 led to decreased EGFR phosphorylation (Fig. 5D), which phenocopied the modulating function of SNX1 on EGFR signaling (14). Taken together, these results proved that miR-95 promotes CRC cell proliferation via directly targeting SNX1.

### Discussion

Knowledge about the precise molecular mechanisms underlying colorectal tumorigenesis is crucial in the develop-

ment of better therapy strategy for CRC patients. Recent advances have suggested that dysregulation of miRNAs is a common event in human cancers, including CRC (3). In this study, we revealed that a panel of 49 miRNAs was altered in CRC tissues. Of these, 49 differentially expressed miRNAs, miR-135b (12), -145 (11), -183 (22), -182 (23, 24), -96 (24), -195 (25), -204 (26), -221 (27), -224 (28), -1 (29), and miR-17-92 cluster (30–32) have been reported in CRC (11, 12, 32) and other cancers (22–29). These data suggest that these miRNAs may have common roles on tumorigenesis. Based on our preliminary functional tests, we focused on miR-95, which had been reported to be up-regulated in CRC (16, 17), lung cancer (18), and pancreatic cancer (19, 20). However, no information about the function or molecular mechanism of miR-95 in human diseases had been reported. In this study, we found that miR-95 is frequently up-regulated in CRC tissues, and that overexpression of miR-95 can significantly promote CRC cell proliferation both *in vitro* and *in vivo*. Moreover, we identified SNX1, a putative tumor suppressor in CRC (14), as a direct functional target of miR-95.

Mammalian SNXs are a group of highly diverse proteins defined by the presence of a phospholipid-binding domain



**Figure 4.** Expression of SNX1 is often decreased and inversely correlated with miR-95 expression in human CRC tissues. **A**, immunohistochemical staining of SNX1 in tumor tissue and corresponding normal colonic epithelium (a, 100 $\times$ ). Normal epithelium shows vesicular brown cytoplasmic SNX1 staining (c, 400 $\times$ ). This staining is nearly entirely absent from the tumor tissue (b, 400 $\times$ ). **B**, SNX1 expression is frequently decreased in tumor tissues when compared to the matched noncancerous tissues. **C**, the expression levels of SNX1 in tumor tissues correlated inversely with the miR-95 expression levels (Spearman  $r = -0.197$ ,  $P = 0.017$ ).

termed the phox homology domain (33). Increasing evidence points to a central role of SNXs for vesicle trafficking processes in oncogenesis and tumor suppression, and SNX1, SNX2, SNX10, and SNX16 have been shown to be involved in tumorigenesis (14, 34–37). SNX1 is the mammalian homologue of the yeast vacuole protein-sorting molecule, Vps5p, and is implicated in endosome-to-lysosome sorting of cell surface receptors, including multiple receptor tyrosine kinases (EGFR, PDGFR, the insulin receptor, and the transferrin receptor; ref. 38), G protein-coupled receptors (protease-activated receptor-1; ref. 39),

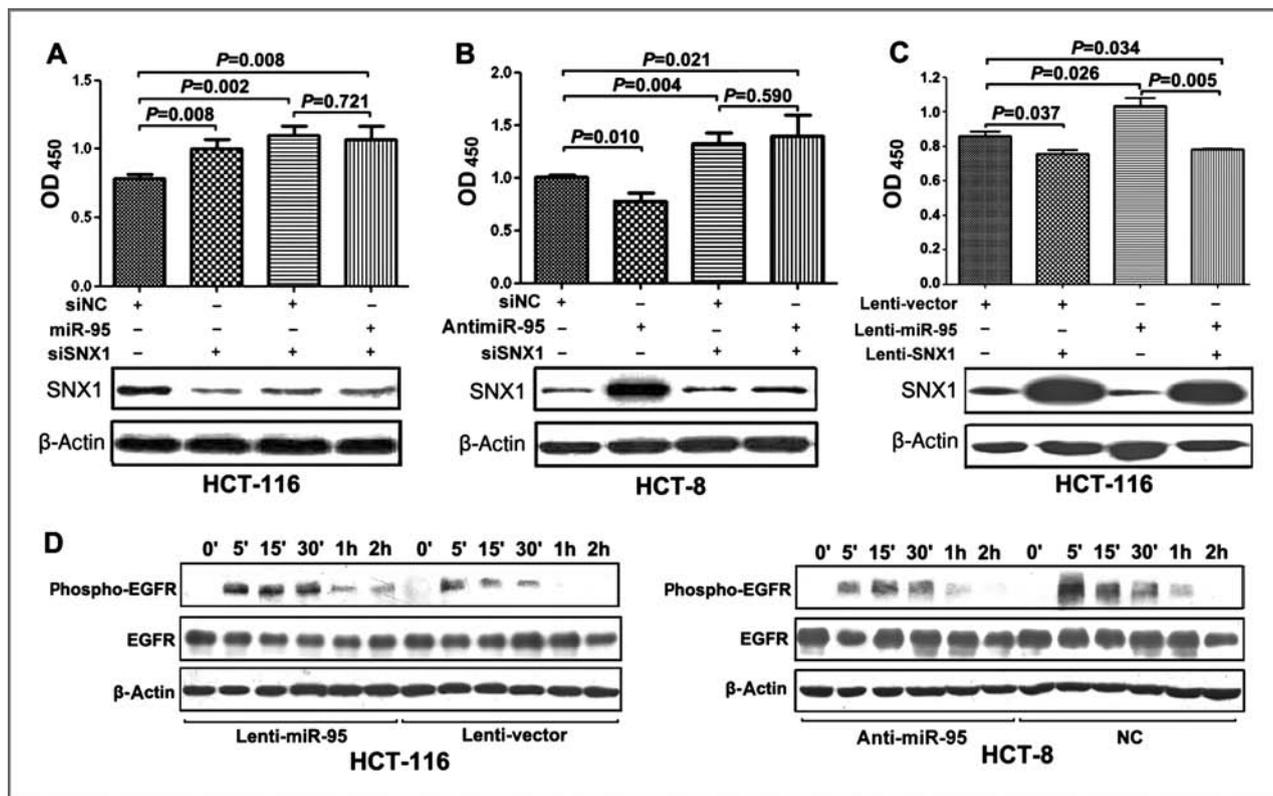
and multiple receptor serine–threonine kinases (TGF- $\beta$ ; ref. 40). Nguyen and colleagues (14) showed that the protein levels of SNX1 were significantly down-regulated in 75% (15/20) of human CRCs. Consistent with these past findings, our results from a large cohort also showed that SNX1 was significantly down-regulated in the majority of human CRCs (78%, 114/146). However, the mechanism of SNX1 underexpression in CRC has not yet been elucidated. This is the first line of evidence to support the hypothesis that underexpression of SNX1 in CRC may result from overexpression of a specific miRNA molecule, miR-95,

**Table 1.** The expression levels of SNX1 in CRC tissues

miR-95 levels <sup>a</sup>	SNX1 protein expression <sup>b</sup>				Total
	Score 0	Score 1	Score 2	Score 3	
Low	21 (28.8%)	28 (38.4%)	12 (16.4%)	12 (16.4%)	73
High	35 (47.9%)	20 (27.4%)	5 (6.8%)	13 (17.8%)	73
Total	56 (38.4%)	48 (32.9%)	17 (11.6%)	25 (17.1%)	146

<sup>a</sup>The median value of miR-95 levels in 146 CRC tissues is used to divide these cases into 2 groups with low or high miR-95 levels, respectively.

<sup>b</sup>The staining intensities are scored and represented as follows: 0, no staining or faint cytoplasmic blush in less than 25% of tumor cells; 1, faint cytoplasmic blush in more than 25% of tumor cells; 2, vesicular pattern no more intense than adjacent normal epithelium; 3, vesicular pattern more intense than adjacent normal epithelium.



**Figure 5.** MiR-95 promotes CRC cell growth via downregulating SNX1 expression. **A**, knockdown of SNX1 by siRNA significantly promoted CRC cell proliferation; and overexpression of miR-95 could not promote cell proliferation in SNX1-depleted HCT-116 cells. **B**, knockdown of miR-95 restored endogenous SNX1 protein expression and inhibited CRC cell growth, but could not repress cell proliferation in SNX1-depleted HCT-8 cells. **C**, SNX1 could significantly abrogate miR-95-induced cell growth. Cell proliferation assays of HCT-116 stable cells were performed after transduction by lenti-SNX1 or vector control lentivirus. Cell proliferation analysis was performed 96 hours after transfection and SNX1 protein expression was detected 48 hours after transfection (**A–C**). **D**, increased EGFR phosphorylation in miR-95-overexpressing CRC cells (HCT-116) and decreased EGFR phosphorylation in miR-95-depleted CRC cells (HCT-8). Cells were starved in serum-free medium for 24 hours and then were treated for the indicated times with 20 mg/L EGF. Activation of EGFR was evaluated using a phosphorylation-specific antibody against Tyr1068.

indicating a new regulating mechanism for vesicle sorting to drive proliferation of CRC cells.

In summary, we have identified an important proliferation-promoting miRNA, miR-95, which was frequently overexpressed in human CRC. Re-expression of miR-95 promoted CRC cell proliferation through negative regulation of SNX1 expression. Expanding insights into the key of miRNA dysregulation implicated in colorectal tumorigenesis will yield important clues to improve our understanding of the complicated molecular pathogenesis of CRC and may enhance the development of new therapeutic regimens for CRC patients.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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