MicroRNA-552 links Wnt signaling to p53 tumor suppressor in colorectal cancer

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Abstract. The aberrant expression of microRNAs (miRNAs or miRs) has been shown to be associated with the development of various types of cancer, including colorectal cancer (CRC). The increased activation of the Wnt signaling pathway via the loss of the Wnt repressor, adenomatous polyposis coli (*APC*), is the hallmark of human CRC. In this study, we demonstrate that the activation of the Wnt/c-Myc axis inhibits the expression of the tumor suppressor, *p53*, via promoting the targeting of p53 by miR-552. Our results revealed that the ectopic expression of miR-552 enhanced cell proliferation, colony formation and resistance to drug-induced apoptosis, suggesting that this miRNA may function as an oncogene. We found that miR-552 displayed oncogenic properties by directly targeting the *p53* tumor suppressor. Of note, our genetic and pharmacological experiments revealed that the Wnt/β-catenin signaling pathway and its major downstream target, c-Myc (hereafter termed Myc), increased the miR-552 levels, and chromatin immunoprecipitation (ChIP) assays revealed they carried out this function by directly binding to their binding sites in the miR-552 promoter region. Given that the functional loss of *APC*, leading to abnormal Wnt signals, and the absence of p53 protein are common in CRC, these results suggest that miR-552 may serve as an important link between these two events, and this warrants further investigation. Collectively, the data of this study suggest that the inhibition of miR-552 may disconnect elevated Wnt signals from p53 suppression, providing a novel therapeutic strategy for patients with CRC with deregulated Wnt signaling.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed type of cancer and the second most common cause of cancer-related mortality worldwide (1). The highest incidence of CRC has been reported in Korea and the number of patients with CRC is increasing each year. The causes of this disease are very diverse, from Westernized diets to genetic factors. CRC development is a multistep process, including the accumulation of epigenetic alterations and genetic abnormalities, such as mutations in tumor suppressor genes or oncogenes (2).

Wnt signaling is critical for animal developmental processes, such as tissue regeneration and morphogenesis (3). There are two distinct pathways in Wnt signaling: Canonical and non-canonical. Signaling components of the canonical Wnt pathway include adenomatous polyposis coli (APC), axin, glycogen synthesis kinase 3β (GSK3β) and β-catenin, while the non-canonical Wnt pathway is regulated by small G protein or Ca^{2+} (4). The hyperactivation of Wnt and thus the nuclear accumulation of β-catenin are frequently observed in CRC (5). In the absence of Wnt ligands, the destruction complex that is composed of APC, Axin, GSK3β, casein kinase 1 (CK1) and other components captures β-catenin in the cytoplasm. β-catenin is then phosphorylated by the destruction complex and is ubiquitinated, followed by proteasomal degradation. In the presence of Wnt ligands, the Frizzled receptor and lipoprotein-related protein 5/6 (LRP5/6) receptor are activated. The LRP5/6 receptor is phosphorylated and recruits the destruction complex. Stabilized β-catenin translocates from the cytoplasm to the nucleus and interacts with TCF/LEF to activate the transcription of Wnt target genes (6). The aberrant activation of the Wnt/β-catenin pathway has been shown to be a central oncogenic driver in CRC by upregulating Wnt target genes, such as *Myc* and *cyclin D1* (7,8). These genes are associated with cell survival and proliferation, and thus may possibly contribute to the development of CRC (9).

The tumor suppressor protein, p53, encoded by *TP53*, is a key regulator of genomic stability, apoptosis, cell cycle and angiogenesis (10). p53 becomes activated upon various cellular stresses, such as oxidative stress, ribonucleotide depletion, deregulated oncogene expression and DNA damage (11). When DNA is damaged, p53 activates the DNA repair system to restore damaged DNA, while terminating the cell cycle progression at checkpoints (12). However, when the damage is irreparable, p53 initiates apoptosis by activating the transcription of its target genes, including *BAX, PUMA* and *Fas* (also known as *APO1*) (13). Wild-type p53 is a labile protein with a half-life of ~5-20 min in most cell types that increases

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by several fold following DNA damage (14). Mouse double minute 2 homolog (MDM2) is a major negative regulator that contributes to the instability of p53 through ubiquitination and the subsequent proteasomal degradation of p53 (15).

The inactivation of the *p53* tumor suppressor gene occurs in the majority of human cancers, suggesting that the loss of p53 function is a critical step in tumorigenesis. It is estimated that loss-of-function mutations in this gene occur in approximately 40-50% of patients with CRC, and the loss of p53 is considered a late event during the transition from adenoma to carcinoma (16,17). Several observations are reminiscent of a crosstalk between the dysregulation of Wnt signaling and p53 dysfunction: i) The strong nuclear accumulation of β-catenin is associated with a high frequency of *p53* mutations in human CRC; ii) there is an inverse correlation between the levels of β-catenin and p53 in a variety of cell types; and iii) activated p53 reduces β-catenin levels via the stimulation of GSK3β (18). Intriguingly, it has recently been shown that the C-terminus of β-catenin inhibits p53 acetylation and its transcriptional activity in smooth muscle cells (19). However, the regulation of p53 levels by Wnt signaling is not yet fully understood in CRC.

MicroRNAs (miRNAs or miRs) are a class of non-coding RNAs and conserved families of transcripts approximately 18-22 nucleotides (nt) in length (20). miRNAs have been found in animals, plants and viruses (21). In animals, they are generated from large hairpin precursors (pri-miRNA) and processed to pre-miRNAs by nuclear RNase III Drosha in the nucleus. pre-miRNAs are exported by exportin-5 (Exp5) from the nucleus to the cytoplasm and are cleaved by cytoplasmic RNase III Dicer to produce mature miRNAs with the aid of AGO (22). In plants and viruses, the mechanism of miRNA processing is similar, although some of the proteins involved are different.

The majority of known miRNAs have been reported to originate from non-protein-coding regions, such as intergenic regions and intronic regions (20,21). Previous studies have identified transposable elements (TEs) as important sources of miRNAs, contributing to >10% of the human miRNA genes (23,24). TEs can be classified into two different groups according to their mode of transposition: Class I TEs (retrotransposons) and class II TEs (DNA transposons). The former is transcribed into RNA that is reverse transcribed into DNA and the copied DNA is inserted into a new site in the genome, while the latter is cut and pasted into a different position (25). Retrotransposons consist of two major groups, long terminal repeats (LTR) and non-LTR retrotransposons, depending on the presence of an LTR at each end. Non-LTR retrotransposons can be subdivided into long and short interspersed elements (LINEs and SINEs). It has been reported that LINEs comprise approximately 17% of the human genome; however, only a small percentage is active and capable of retrotransposition. Although LINEs are autonomous and can transpose autonomously, SINEs are non-autonomous and depend on the machinery of other retrotransposons for transposition (25).

miRNAs play important roles in diverse biological processes, such as apoptosis, organ development and cell proliferation (26). They mostly downregulate gene expression by base pairing to 3'-or-5'-untranslated regions (UTRs) in target mRNAs, which can result in the inhibition of target gene translation or promotion of mRNA degradation (27). It has been shown that the aberrant expression of miRNAs is associated with the development of various types of cancer, including CRC (28), and these miRNAs affect different aspects of colon carcinogenesis (29). The targeting of E2F1 by miR34a, that of Rb by miR-675, and that of Ras by miR-143 and let-7 inhibit the proliferation of CRC cells; miR-26, miR-145 and miR-196a regulate invasiveness and metastasis; miR-107 induced by p53 inhibits tumor angiogenesis, the process of new blood vessel development, by blocking the hypoxia-inducible factor (HIF) 1-mediated expression of vascular endothelial growth factor (VEGF); and the levels of miR-17-3p and miR-92a in plasma samples from patients with CRC have been found to be markedly elevated and these miRNAs may potentially be developed into novel diagnostic markers.

Previous studies have indicated that the dysregulation of miR-552 is linked to increased Wnt signaling in CRC cells (30,31). Given the frequent inactivating mutations in *APC* and abnormal Wnt signals, in this study, we further characterized the association between miR-552 and the Wnt pathway. We hypothesized that miR-552 acts as an oncogene and mediator that transduces the activation of Wnt signaling to the downregulation of the p53 tumor suppressor. Revealing the underlying mechanism of the aberrations in miR-552 expression and functions may provide novel therapeutic targets for CRC.

Materials and methods

Cell culture, antibodies and reagents. The human CRC cell lines HCT116 (10247; Korean Cell Line Bank, Seoul, Korea) and DLD-1 (10221; Korean Cell Line Bank) were cultured in DMEM (Welgene, Gyeongsan, Korea) and RPMI-1640 medium (Gibco/Thermo Fisher Scientific, Waltham, MA, USA), respectively, supplemented with 10% fetal bovine serum (FBS, Capricorn Scientific, Ebsdorfergrund, Germany), 1% N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1% L-glutamine and 1% penicillin/streptomycin (PEN/STR) at 37˚C in a humidified 5% CO_2 incubator.

The following primary antibodies were used in this study: Anti-β-actin [1:5,000 dilution; sc-47778; Santa Cruz Biotechnology (SCB), Dallas, TX, USA], anti-phospho-GSK3β ser9 (1:2,000 dilution; 5558; Cell Signaling Technology, Danvers, MA, USA), anti-TCF4 (1:1,000 dilution; 05-511; MilliporeSigma, Burlington, MA, USA), anti-β-catenin (1:2,000 dilution; sc-7199; SCB), anti-p53 (1:1,000 dilution; sc-126; SCB), anti-Myc (1:2,000 dilution; ab32072; Abcam, Cambridge, UK) antibodies. HRP-conjugated anti-rabbit (A120-101p) and -mouse (A90-116p-33) secondary antibodies were from Bethyl (1:5,000 dilution; Montgomery, TX, USA).

The chemicals used in this study were doxorubicin (sc-200923; SCB), LiCl (L4408; Sigma, St. Louis, MO, USA), a BET bromodomain inhibitor JQ1 (A1910; APExBIO; Boston, MA, USA), a Myc-Max dimerization inhibitor 10058-F4 (475956; MilliporeSigma, Burlington, MA, USA), crystal violet (C0775; Sigma).

Reverse transcription-quantitative PCR (RT-qPCR). The transcription level of miR-552 was measured by RT-qPCR as previously described (32). Total RNA was extracted using

TRIzol reagent (Favorgen, Ping-Tung, Taiwan) and reverse transcription was performed to synthesize cDNA using the High Capacity cDNA Reverse Transcription kit, TaqMan MicroRNA Assay Probes and Universal Master Mix II (Applied Biosystems, Foster City, CA, USA). The thermocycling condition for RT-qPCR were 10 m at 95˚C for the initial denaturation followed by 39 cycles of 15 sec at 95˚C and 60 sec at 60˚C. Relative gene expression was analyzed using the $2^{-\Delta\Delta Cq}$ method (33).

To measure the transcriptional level of LINEs, the PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time) (Takara, Shiga, Japan) was used to synthesize the cDNA. qPCR was conducted using TOPreal™ qPCR 2X PreMIX (SYBR-Green with low ROX) (Enzynomics, Daejeon, Korea) and target primers. The thermocycling condition for qPCR were 15 m at 95˚C for the initial denaturation followed by 40 cycles of 15 sec at 95˚C, 15 sec at 60˚C and 30 sec at 72˚C and a final extension for 10 sec at 95˚C. The sequences for the TBP-, LINE- and Myc-specific primers were as follows: TBP forward, 5'-TATAATCCCAAG CG GTTTGC TGCG-3' and reverse, 5'-AATTGTTGGTGGGTGAGCACA AGG-3'; LINE forward, 5'-CAAATCACACACCTGAAAA GGA-3'andreverse,5'-CATGGTTTTAATTTGCATTACCC-3'; Myc forward, 5'-CTCCTGGCAAAAGGTCAGAG-3' and reverse, 5'-TCGGTTGTTGCTGATCTGTC-3'.

Western blot analysis. To perform western blot analysis, relevant cells at a density of two million cells per well in 6-well plates were washed with PBS and lysed in lysis buffer [Protein Extraction Solution (Elpis Biotech, Daejeon, Korea), Na-vanadate (1 mM), β-glycerol phosphate (50 mM), protease inhibitor (G-Biosciences), EDTA (5 mM) and β-mercaptoethanol (142.7 mM; Bioworld, Irving, TX, USA)]. The samples were boiled at 100˚C for 10 min and loaded on 10% polyacrylamide gels. Twenty micrograms of proteins were transferred onto membranes using the Mini Trans-Blot® Cell and Criterion™ Blotter (Bio-Rad, Hercules, CA, USA), and membranes were blocked in 1% BSA dissolved in Tris-buffered saline containing 0.1% Tween-20 (TBST). The membranes were probed with the indicated primary antibodies overnight at 4˚C, washed for 5 min in TBST, and incubated with anti-mouse or anti-rabbit secondary antibodies for 1 h at room temperature. After washing 3 times for 10 min each with TBST at room temperature, the bands were detected with a chemiluminescent substrate (Ez West Lumi Plus, ATTO Technology, Amherst, NY, USA) and visualized on the Chemiluminescence Imaging system (Luminograph II, ATTO Technology).

Transfection of short interfering RNA (siRNA), short hairpin RNA (shRNA) and expression vectors. A total of 100 pM of Myc siRNA (sense, 5'-GACAGUGUCAGAGUCCUGA-3' and antisense, 5'-UCAGGACUCUGACACUGUC-3'), 100 pM of a non-targeting negative control siRNA (Bioneer, Daejeon, Korea), 1 μg of a non-targeting shRNA control vector, or 1 μg of shRNAs targeting β-catenin or TCF4, 1 μg of empty pcDNA control vector, or 1 μg of pcDNA-Myc vector (please see the Acknowledgements section below) were transfected into two million HCT116 cells in 6-well plates using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) (34), followed by incubation for 24 h. Similar transfection efficiencies were confirmed by FACS analysis of the expression of green fluorescent protein (GFP).

Stable and transient expression of miR-552. To generate HCT116 and DLD-1 cells stably expressing miR-552, a fragment (~300 bp) containing the miR-552 sequence was PCR-amplified using primers as follows: Forward, 5'-TTTTTAGATCTAAACCCAG CATGCCTATGAC-3' and reverse, 5'-TTTGAATTCCCTCC ACCTCACCACATTCT-3'. The PCR product was directionally cloned into the *Bgl*II and *Eco*RI sites of the murine stem cell virus (MSCV)-puro retroviral vector. Retrovirus was generated by co-transfection of the MSCV constructs with pKAT and VSVG (vesicular stomatitis virus G glycoprotein), which were a kind gift from Dr R. Aguiar (University of Texas Health Science Center at San Antonio, TX, USA), into 293 cells (21573; Korean Cell Line Bank) using Lipofectamine 2000 (Invitrogen). At 48 h following transfection, cells were infected with the viral supernatant and selected using puromycin (1 mg/ml). For the transient expression of miR-552, the HCT116 cells were transfected with 100 pM of miR-552 mimic or miRNA negative control (Bioneer, Daejeon, Korea) using Lipofectamine 2000 (Invitrogen).

Cell counting and colony forming assays. For cell counting, two million cells were seeded in a 6-well cell culture plate (SPL Life Sciences, Pocheon, Korea) at day 0 and counted every 24 h. For colony forming assays, miR-552-expressing cells or control cells were seeded in a 6-well plate (1,000 cells/well), and 1 week later colonies were fixed and stained with 0.5% crystal violet at room temperature for 10 min.

Chromatin immunoprecipitation (ChIP) assays. ChIP assays were performed as previously described (35). Briefly, approximately $1.0x10^7$ to $2.0x10^7$ HCT116 cells were harvested and proteins were cross-linked to DNA with 1% formaldehyde, followed by fixation and sonication to shear the DNA to an average size of ~300 nucleotides. The fragmented chromatin was reacted with antibodies in a volume of 1,000 μl and immunoprecipitated with protein A/G agarose beads (sc2003; SCB). The immune complexes were washed 5 times before eluting the DNA. The primary antibodies used were anti-Myc antibody (sc764: SCB), normal rabbit IgG (sc2027; SCB), anti-TCF4 antibody (05-511; MilliporeSigma), and normal mouse IgG (12-371; MilliporeSigma). The obtained DNA was analyzed by RT-qPCR using the primers described below. Primers for amplifying the 3 potential TCF4 binding sites in the miR-552 promoter were designated as 1, 2, and 3: Myc forward, 5'-TTTTGCTAGCTCAAAACTCAATGGTAAA-3' andreverse,5'-TTTTCTCGAGACAATTTTACATCCCATC-3'; TCF4 forward 1, 5'-TTCTTTTTTCCAAATTGATTCAAA CAT-3' and reverse 1, 5'-TCCACCCTCTTCTTCTTTAGC-3'; TCF4 forward 2, 5'-GGGCTTTTACGGATGTCAGA-3' and reverse 2, 5'-GACAGGCTCCTGGATTGAAG-3'; TCF4 forward 3, 5'-CTCCCACTTGTCACCAGCTC-3' and reverse 3, 5'-GGCAGTAGGAGAGGGAGAGAG-3'.

p53 3'-UTR luciferase reporter assays and site-directed mutagenesis. The 3'UTR of p53 with a probable miR-552 binding

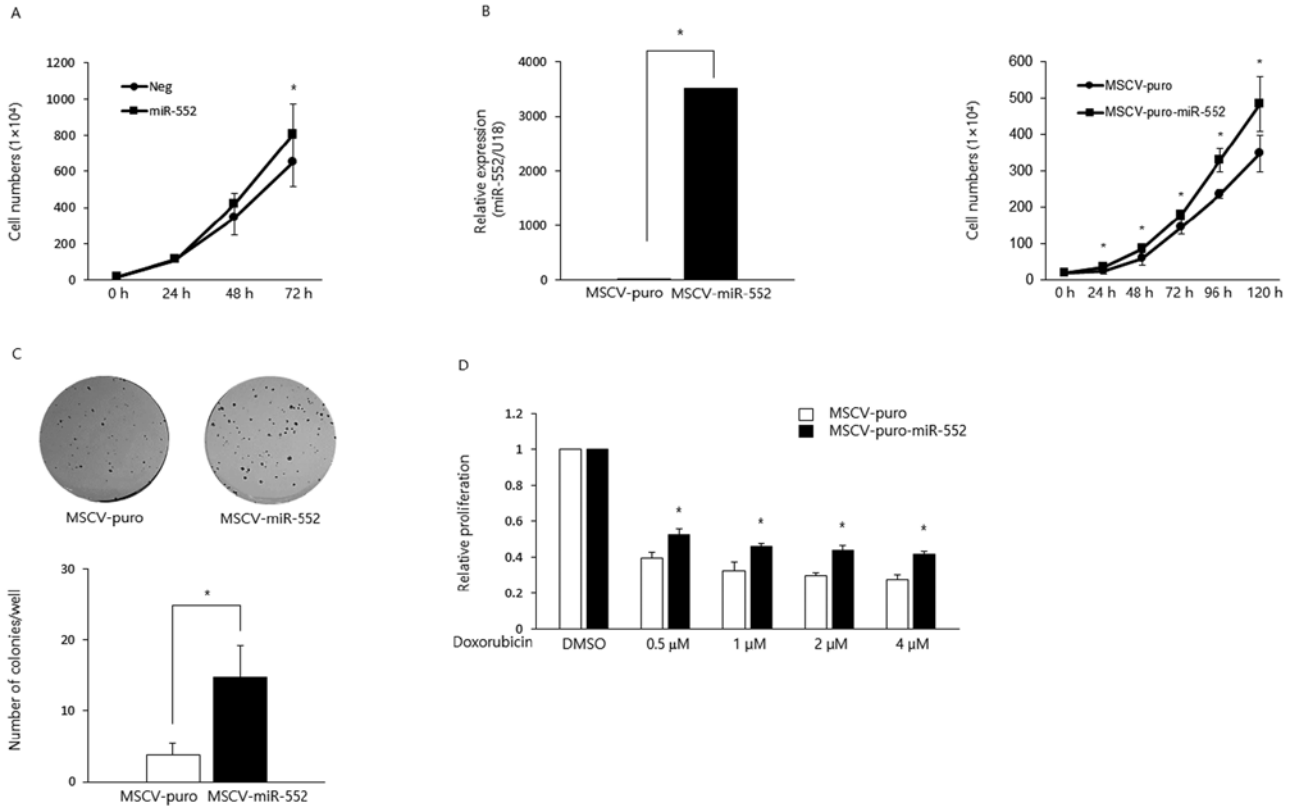


Figure 1. miR-552 functions as an oncogene. (A) The effect of the transient expression of miR-552 on HCT116 cell proliferation was analyzed. The number of cells was counted every 24 h, following transfection of the control or miR-552 mimics. The transient expression of miR-552 enhanced proliferation (*P<0.05). (B) HCT116 cells were transduced with MSCV-puro or MSCV-puro-miR-552 retroviral constructs. The stable expression of MSCV-puro-miR-552 expression was confirmed by RT-qPCR (left panel; *P<0.05). The ectopic expression of miR-552 increased cell proliferation (right panel; *P<0.05). (C) Colony formation assays were performed with HCT116 cells ectopically expressing the vector control or miR-552. The cells were seeded in 6-well plates and cultured for 7 days, followed by crystal violet staining and the quantification of colonies. Representative images of colony formation are shown in the upper panel. Colony formation was enhanced by the ectopic expression of miR-552 (*P<0.05). (D) MSCV-puro and MSCV-puro-miR-552 cells were treated with doxorubicin (0, 0.5, 1, 2 and 4 μM) and counted every 24 h. The expression of miR-552 rendered the cells resistant to doxorubicin-induced cell death (*P<0.05). All experiments were performed at least 3 times independently and a representative result is shown.

site was cloned into the psiCHECK-2 vector (Promega, Madison, WI, USA) using the following primers: Forward, 5'-TTTCTCGAGGAGACTGGGTCTCGCTTTGT-3' and reverse, 5'-TTTGCGGCCGCAAATGCAGATGTGCTTG CAG-3'. The putative miR-552 binding site was mutated using a QuikChange Site-Directed Mutagenesis kit (Stratagene, Santa Clara, CA, USA). Primers for mutagenesis were as follows: Forward, 5'-GGCTCAGGCGATCGAGGTCTCTC AGCCTCCCAG-3' and reverse, 5'-CTGGGAGGCTGAGA GACCTCGATCGCCTGAGCC-3'. Sanger sequencing was performed to verify the sequences of the mutated construct. For luciferase assays, 500 ng of p53 3'-UTR construct with the wild-type or mutant miR-552 binding site were co-transfected with 20 pM of miR-552 mimic or control oligonucleotides (Bioneer, Seoul, Korea). The cells were harvested at 72 h post-transfection and dual luciferase reporter assays were performed (Promega).

Statistical analysis. The data are presented as the means ± standard deviation (SD). Statistical analyses were performed using a non-parametric Mann-Whitney U test to identify statistically significant differences (P<0.05). Prism software was used to perform the statistical analysis. All experiments were performed at least 3 times independently.

Software programs. The software programs used in this study are TRANSFAC version 8.0 (genexplain/transfac) to identify potential transcription factor-binding sites, TargetScan Version 7.1 (www.targetscan.org) to analyze the targets of miRNAs, and STarMir (http://sfold.wadsworth.org/cgi-bin/starmirtest2.pl) to predict miRNA binding sites.

Results

miR-552 is an oncogene in CRC. A previous study analyzed the expression of miR-552 in 183 pairs of non-cancerous colon samples and CRC tissues and found a significantly higher expression of this miRNA (36), which suggests that miR-552 acts as an oncogene in CRC. To directly test this, in this study, we transiently overexpressed this miRNA in HCT116 CRC cells and found that the ectopic expression of miR-552 increased cell proliferation (Fig. 1A). To further characterize its oncogenic potential, we generated HCT116 stable cell lines to ectopically express MSCV-puro or MSCV-puro-miR-552 using retroviral constructs (Fig. 1B, left panel). The overexpression of miR-552 promoted cell proliferation (Fig. 1B, right panel) and colony formation (Fig. 1C). Moreover, miR-552-expressing cells exhibited resistance to the doxorubicin-induced suppression of proliferation (Fig. 1D).

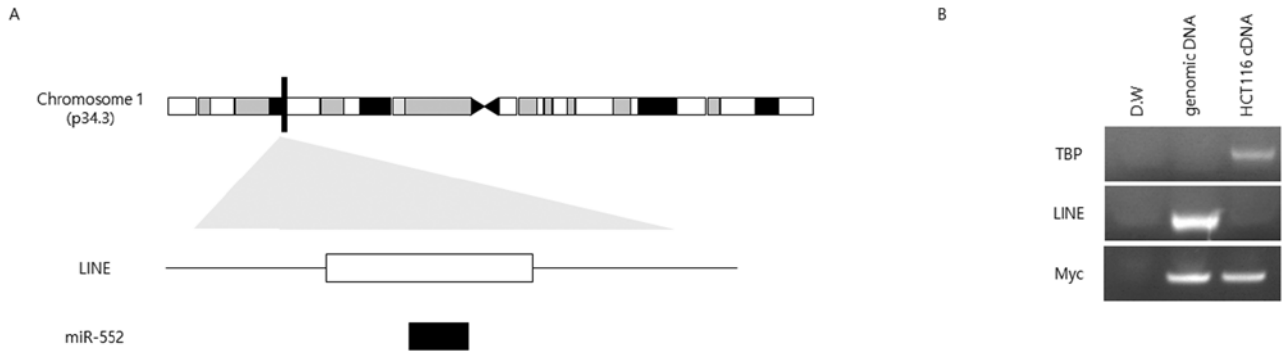


Figure 2. miR-552 is located in a long interspersed nuclear element (LINE). (A) A schematic diagram of the location of miR-552 in chromosome 1. miR-552 is overlapped with a LINE sequence. (B) RT-qPCR was performed using the PrimeScript™ RT reagent Kit with gDNA Eraser and TOPreal™ qPCR 2X PreMIX to examine whether the LINE sequence was producing mRNA products. Genomic DNA from OCI-Ly1 B lymphoma cells was used as negative (-) and positive (+) controls for TATA-box binding protein (TBP) and LINE-specific primers, respectively. Myc-specific primers were used as an internal control. This LINE was not active.

miR-552 is located in a LINE. Our sequence analysis suggested that the sequences of miR-552 and LINE L1 overlapped (Fig. 2A); therefore, we evaluated whether the expression of miR-552 is affected by that of L1. To that end, we designed primers for specifically amplifying the LINE L1 sequence to test whether this specific LINE was active or produced RNA transcripts. No PCR product was detected when cDNA was used as a template, whereas genomic DNA as a positive control produced a single band of the expected size (Fig. 2B).

miR-552 is directly regulated by the Wnt-Myc axis. To investigate the transcriptional control of miR-552, we identified transcription factors that potentially bind to the promoter using TRANSFAC software (Fig. 3A). Among the many TFs that may influence miR-552 transcription, we were interested in characterizing the TCF4- and Myc-binding sites as the inactivation of APC, thereby the hyperactivation of Wnt signaling, and the dysregulation of Myc (8), a critical downstream target gene of the Wnt pathway, are frequently observed in CRC. We wished to determine whether the miR-552 levels were affected by Wnt signaling. GSK3β, a component of the destruction complex, has been known to catalyze the phosphorylation and ubiquitination of β-catenin in the absence of Wnt protein, marking it for degradation by the proteasome (6). LiCl is a well-characterized inhibitor of GSK3β, and its addition in HCT116 cells led to an increase in the phosphorylation of GSK3β (ser9) and concomitant increase of miR-552 (Fig. 3B). To further elucidate the role for the Wnt signaling in the regulation of miR-552 expression, we genetically silenced β-catenin and its binding partner, TCF4, using shRNA constructs, which markedly inhibited the expression of this miRNA, suggesting that the Wnt pathway regulates the expression of miR-552 (Fig. 3C).

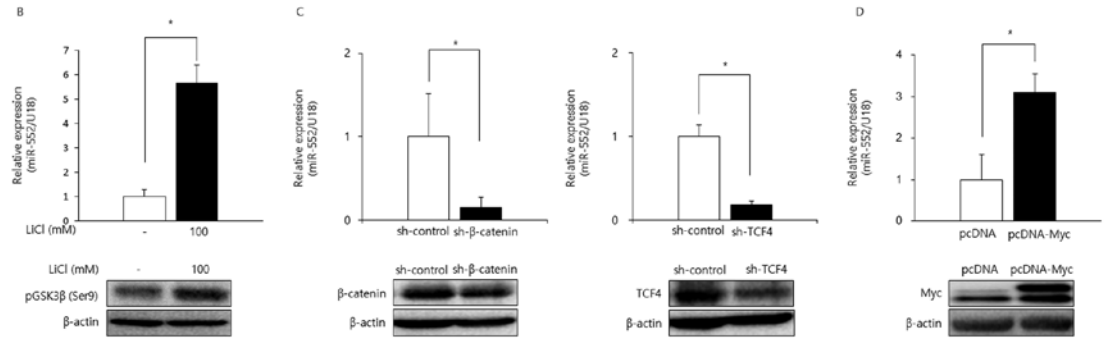
Subsequently, we investigated whether the miR-552 levels would be modulated by Myc in CRC. The ectopic expression of Myc in HCT116 cells resulted in the upregulation of miR-552 (Fig. 3D). Consistently, genetic knockdown using Myc-targeting siRNAs or the pharmacological inhibition of Myc using JQ1, a BET bromodomain inhibitor, or 10058-F4, a Myc-Max dimerization inhibitor, downregulated the expression of miR-552 (Fig. 3E-G).

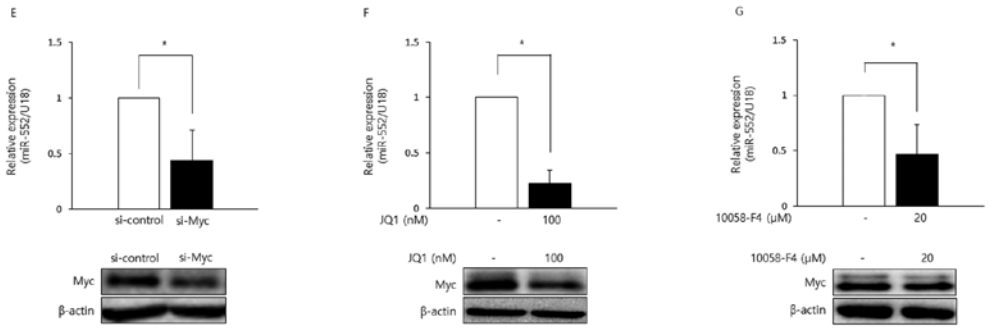
Given that the miR-552 levels were regulated by the Wnt/Myc axis, we wished to delineate the underlying mechanisms. We suspected that the regulation of miR-552 by Myc and the Wnt signals was accomplished via the Myc E-box and TCF4-binding sites in the promoter region of this miRNA. To directly investigate whether the Myc E-box and TCF4-binding sites were functional, we performed ChIP assays using normal IgG, anti-Myc, or anti-TCF4 antibodies. The promoter region of miR-552 containing the Myc E-box was enriched by antibodies against Myc, suggesting that Myc directly binds to the E-box in the miR-552 promoter (Fig. 3H). Anti-TCF4 antibodies efficiently immunoprecipitated one of the three probable TCF4-binding sites, indicating that only one, but not the other two, TCF4-binding site was functional (Fig. 3I). Taken together, these data clearly demonstrated that miR-552 was directly upregulated by Myc and TCF4 transcription factors via their corresponding binding sites in the miR-552 promoter region.

The p53 tumor suppressor is a target of miR-552. The results described above clearly demonstrated that elevated miR-552 levels promote the oncogenic properties of CRC cells. Based on these data, we predicted that miR-552 inhibits the expression of tumor suppressor genes and aimed to identify a potential target of this miRNA relevant in our experimental setting. The use of miRNA binding site prediction algorithms (TargetScan version 7.1) revealed that the *p53* tumor suppressor gene has a potential binding site within its 3'-UTR. The putative binding site was found only in humans and chimps (Fig. 4A), suggesting that the interaction between these two genetic elements came late in the evolution. To examine whether miR-552 directly binds to the potential binding site in the p53 3'-UTR, we cloned p53 3'UTR in the wild-type or mutant configuration in the psiCHECK-2 luciferase vector and reporter assay was performed (Fig. 4B). The luciferase activity of the wild-type construct was efficiently repressed by miR-552. However, miR-552 did not affect luciferase reporter activity when the binding site was mutated, suggesting miR-552 directly interacts with its binding site in the p53 3'-UTR. Next, to investigate whether miR-552 regulates the p53 levels, we generated HCT116 and DLD-1 CRC cells expressing either MSCV-puro or MSCV-puro-miR-552 and examined the p53



Name	Location	Direction	Core match	sequence
TCF4	-3768	(-)	1	atCAAAGa
HNF-1	-3389	(+)	0.874	atgTCAAatttct
HNF4	-3262	(-)	1	ttccaCTTTGcatt
GATA4	-3200	(-)	0.814	tcactagGATTT
HFH-3	-3073	(-)	1	cacacAAACAggt
PolyA	-3000	(-)	0.886	aaagaaaAAGCAcacg
FOXP3	-2976	(+)	0.859	aaagaTTTGTaaca
GATA1	-2822	(-)	1	attgTAATCcctat
MyoD	-2397	(+)	0.932	ctgCAGGTggtc
TCF4	-2059	(+)	1	aCTTTGat
TCF4	-1385	(-)	0.891	atCAAGGg
Myc:Max	-414	(+)	0.941	aaaCACATgaat
MEF-2	-280	(-)	0.903	ataaAAAAAaaa





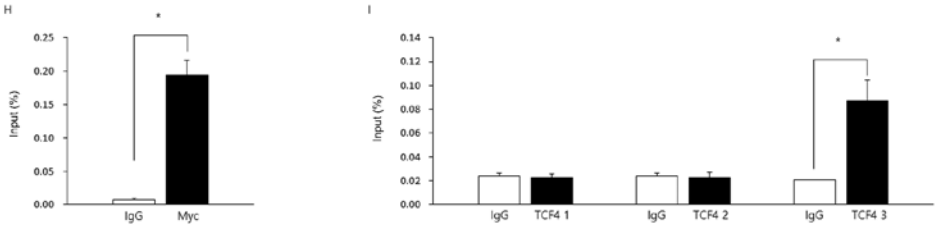


Figure 3. miR-552 is a target of the Wnt/Myc axis. (A) Transcription factor-binding sites in the miR-552 promoter region were identified using TRANSFAC software. Relative positions of TCF4- and Myc-binding sites are shown in the schematic, and the +1 represents the transcriptional start site of miR-552. Direction indicates whether the transcription factor binds to the (+) or (-) strand of the DNA. The core match is defined as the binding probability of transcription factors to their potential DNA binding sites. When the core match for the indicated transcription factor is closer to 1, it has a better chance to bind to the respective binding site. (B) Transcriptional levels of miR-552 were analyzed by RT-qPCR following treatment with LiCl (100 mM, 24 h; upper panel). Phosphorylation levels of the inactive form GSK3β at Ser9 were measured by western blot analysis (lower panel). β-actin was used as a loading control. The inhibition of GSK3β led to increments in miR-552 expression (*P<0.05, non-parametric Mann-Whitney U test). (C) Relative miR-552 transcriptional levels in HCT116 cells were examined by RT-qPCR 48 h post-transfection with an shRNA control vector, or shRNAs targeting β-catenin or TCF4. The downregulation of β-catenin and TCF4 was confirmed by western blot analysis (lower panel) and β-actin was used as a loading control. The inhibition of the Wnt signaling pathway significantly suppressed miR-552 levels (upper panel; *P<0.05). (D and E) A Myc-expressing vector construct or siRNA targeting Myc was transfected into HCT116 cells. Twenty-four hours later, relative miR-552 transcriptional levels were assessed by RT-qPCR. The ectopic expression or knockdown of Myc was confirmed by western blot analysis (lower panel), which led to the up- and downregulation of miR-552 (upper panel; *P<0.05), respectively. β-actin was used as a loading control. (F and G) HCT116 cells were treated with JQ1 (100 nM, 48 h) or 10058F4 (20 μM, 24 h), followed by the analysis of the transcriptional levels of miR-552. Myc levels following treatment with JQ1 or 10058-F4 were analyzed by western blot analysis (lower panel). β-actin was used as a loading control. The inhibition of Myc decreased the expression of miR-552 (upper panel; *P<0.05). (H and I) ChIP assays were performed using anti-Myc and anti-TCF4 antibodies. DNA obtained from the ChIP assay was analyzed by RT-qPCR (upper panel) using primers listed in the Materials and methods. The IgG antibody was used as a negative control. All experiments were performed 3 times independently (*P<0.05, non-parametric Mann-Whitney U test).

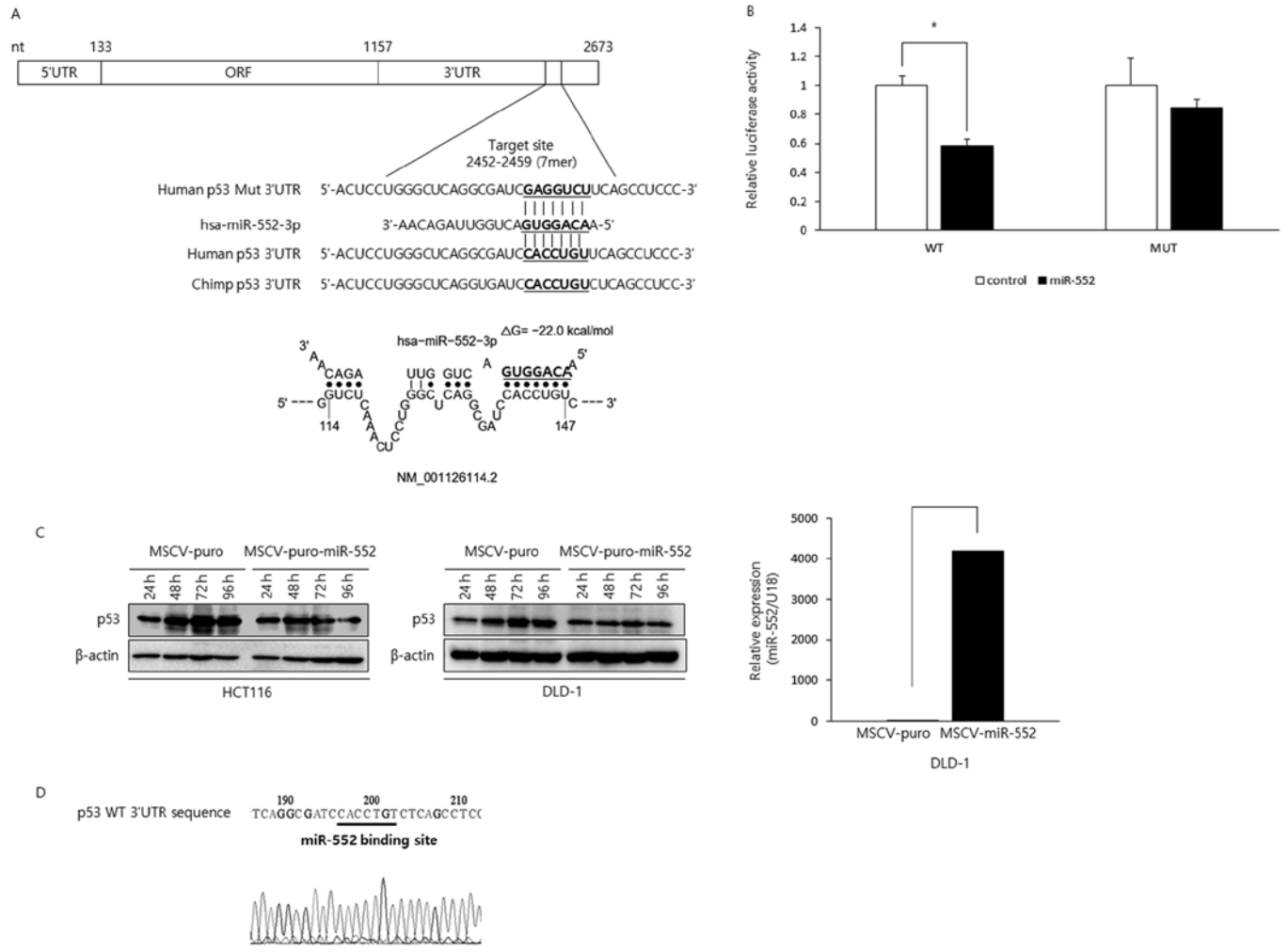


Figure 4. The p53 tumor suppressor is a target of miR-552. (A) miR-552 and the putative binding site in the p53 3' UTR from human and chimpanzee were aligned using TargetScan (version 7.1) and STarMir. ΔG is Gibbs free energy, and spontaneous interaction between miR-552 and its binding site occurs when ΔG<0. The miR-552 binding site was conserved only in human and chimpanzee. (B) The p53 3'-UTR luciferase constructs with a potential miR-552 binding site in the wild-type (WT) or mutant (MUT) configuration were co-transfected with either miR-552 or negative control oligonucleotides. Luciferase reporter assay was performed at 72 h following transfection; luciferase activity of the wild-type, but not mutant, construct was reduced by approximately 40%. (C) HCT116 and DLD-1 MSCV-puro or MSCV-puro-miR-552 cells were plated in the 6-well plates and harvested every 24 h, followed by the analysis of p53 protein levels by western blotting. The p53 levels were increased in MSCV-, but not MSCV-miR-552-expressing, cells (left panel). The ectopic expression of miR-552 in DLD-1 cells was confirmed by RT-qPCR (right panel; *P<0.05, non-parametric Mann-Whitney U test). (D) The potential miR-552-binding site in the 3'UTR in DLD-1 CRC cells were sequenced by the Sanger method. The binding site was intact.

levels by western blot analysis (Fig. 4C). The levels of p53 increased in the control cells in a time-dependent manner. By contrast, the cells expressing miR-552 did not exhibit as much of an increment as the control cells, suggesting that miR-552 suppressed p53 expression. Intriguingly, DLD-1 CRC cells are known to express mutant p53, but our sequencing results indicated that the miR-552 binding site in p53 3'-UTR was intact (Fig. 4D). As expected, the downregulation of p53 by miR-552 was observed in the DLD-1 cells (Fig. 4C). Taken together, these data suggest that miR-552 negatively modulates p53 tumor suppressor in CRC, which is through a direct interaction between miR-552 and its binding site in the p53 3'-UTR.

Discussion

Cancer is a complex disease involving multiple genetic and epigenetic aberrations (1,2). The functional loss of APC, thereby aberrant Wnt signaling, has been found in >80% of CRC samples (3) and it is recognized as one of the earliest genetic events during the development of this type of tumor (37). Another common genetic abnormality observed in CRC is the inactivation of the tumor suppressor p53 signals (16,17). However, the inter-relationship between the Wnt/β-catenin pathway and p53 tumor-suppressive signaling has yet to be fully understood. The data of this study suggest that the activation of Wnt signaling downregulates the expression of p53 via the upregulation of miR-552, a p53-targeting miRNA. This is intriguing as the hyperactivation of Wnt occurs in the early stages of CRC development and may eventually decrease p53 levels, which may help progression to later stages by driving cell proliferation and survival. This offers a unique opportunity where the development of CRC can be interrupted by restoring miR-552 expression. Previous studies have provided clear evidence that miR-34 links p53 tumor suppressor signals to the Wnt pathway (38,39). The expression of miR-34 is increased by p53 (40); miR-34 directly targets

and decreases the expression of some activators of canonical Wnt signaling, such as WNT1, WNT3, β-catenin, LEF1, Axin2 and the Wnt coreceptor LDL receptor-related protein (LRP) 6, which leads to the suppression of Wnt signaling (39); the loss of p53 functions or miR-34 promote CRC progression, which is dependent on the hyperactivation of the Wnt pathway (39). These results revealed that miR-34 is a mediator that transduces the inactivation of p53 tumor suppressor functions to heightened Wnt signaling in cancers. To the best of our knowledge, miR-552 is the first onco-miRNA connecting the hyperactive Wnt pathway with down-modulation of p53 levels, which may contribute to colon carcinogenesis. Previous studies have demonstrated that the loss of p53 function plays a decisive role in carcinogenesis and, indeed, inactivating mutations in p53 have been described in approximately 40-50% of CRCs (16,17). In this regard, the normalization of miR-552 can restore the level of p53 in CRC cells to that in normal cells and may be a feasible therapeutic option for treating patients with CRC driven by high Wnt signaling. The regulation of p53 and miR-552 by the Wnt signals using CRC clinical samples warrants further investigation.

The role of miRNAs in normal cellular physiology has been studied extensively, and the notion that they are dysregulated in a wide range of human diseases, including cancer, has been convincing (41). A previous study demonstrated that miR-552 was highly expressed in CRC samples (36). We thus hypothesized that the expression of miR-552 may be affected by LINE L1 as their sequences overlapped. The present study indicated that this LINE was not active, and thus miR-552 was not expressed as a part of it and that the expression of this miRNA may be controlled by its own promoter (Fig. 2). Our results shed light into the underlying mechanisms of the dysregulation of miR-552 in CRC, i.e., the β-catenin/TCF4 complex and Myc, a major downstream target of the Wnt pathway, directly bind to their corresponding binding sites in the miR-552 promoter region to enhance its expression. Taken together with the results from the previous section, these data suggest that the Wnt-Myc axis can increased the miR-552 levels in HCT116 CRC cells.

Among the identified targets of the miR-552 are the cell fate determination factor dachshund family transcription factor 1 (DACH1) (30) and a disintegrin and metalloprotease (ADAM) family member 28 (*ADAM28*) (31). The inhibition of the expression of these genes by miR-552 increased the oncogenic properties of CRC cells, such as cell proliferation, migration and clonogenicity (30,31) and our study suggests that aberrant Wnt signals may also downregulate these genes by upregulating miR-552. Notably, DACH1 is a negative regulator of the Wnt pathway and the downregulation of DACH1 by miR-552 results in increased Wnt/β-catenin signaling (30). This and the results of the present study indicate the presence of a feedforward loop that may play a critical role in promoting the survival of CRC cells: miR-552 elevates the activity of the Wnt pathway, which in turn increases the expression of miR-552. Given the critical role of the Wnt signaling in normal cellular processes, such as development, this miRNA may be involved in the regulation of normal cell physiology. One of the key findings of this study is the regulation of p53 levels by the Wnt signaling, which is mediated by miR552. Together with previous findings (30), our study indicates the presence of a potential positive feedback loop between the Wnt signals and the levels of p53 tumor suppressor.

In conclusion, the current study demonstrates that miR-552 acts as an oncogene in CRC. Mechanistically, the Wnt/β-catenin signals reduce the expression of p53 tumor suppressor via the upregulation of this miRNA. Previous observations that miR-552 expression was higher in patients with pancreatic cancer and that Wnt signaling was necessary for pancreatic carcinogenesis (42) suggest that this miRNA may be a potential therapeutic target in these types of cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

BK designed and performed the experiments, analyzed the data, and wrote the manuscript; DUK performed the experiments; TOK analyzed the data; HSK supervised the study and analyzed the data; SWK designed and supervised the study, planned experiments, analyzed the data and wrote the manuscript. All the authors have edited and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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