RESEARCH ARTICLE

MiR-133b Acts as a Tumor Suppressor and Negatively Regulates TBPL1 in Colorectal Cancer Cells

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Abstract

Introduction: MicroRNAs have emerged as post-transcriptional regulators that are critically involved in tumorigenesis. This study was designed to explore the effect of miRNA 133b on the proliferation and expression of TBPL1 in colon cancer cells. Methods: Human colon cancer SW-620 cells and human colon adenocarcinoma HT-29 cells were cultured. MiRNA 133b mimcs, miRNA 133b inhibitors, siRNA for TBPL1 and scrambled control were synthesized and transfected into cells. MiR-133b levels in cells and CRC tumor tissue was measured by real-time PCR. TBPL1 mRNA was detected by RT-PCR. Cell proliferation was studied with MTT assay. Western blotting was applied to detect TBPL1 protein levels. Luciferase assays were conducted using a pGL3-promoter vector cloned with full length of 3'UTR of human TBPL1 or 3'UTR with mutant sequence of miR-133b target site in order to confirm if the putative binding site is responsible for the negative regulation of TBPL1 by miR-133b. Results: Real time PCR results showed that miRNA 133b was lower in CRC tissue than that in adjacent tissue. After miR-133b transfection, its level was elevated till 48h, accompanied by lower proliferation in both SW-620 and HT-29 cells. According to that listed in http://www.targetscan.org, the 3'-UTR of TBPL1 mRNA (NM_004865) contains one putative binding site of miR-133b. This site was confirmed to be responsible for the negative regulation by miR-133b with luciferase assay. Further, Western blotting and immunohistochemistry both indicated a higher TBPL1 protein expression level in CRC tissue. Finally, a siRNA for TBPL1 transfection obviously slowed down the cell proliferation in both SW-620 and HT-29 cells. Conclusion: MiR-133b might act as a tumor suppressor and negatively regulate TBPL1 in CRC.

Keywords: microRNA 133b - colon cancer cells - TBP like 1

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Introduction

Colorectal cancer (CRC) is one of the third-most diagnosed cancers and the fourth leading cause of cancer death in the world with an estimated 500,000 deaths annually (Ehrig et al., 2013). Its incidence and mortality in China have increased rapidly in the past few decades (Sun et al., 2013). The rectum (56%) and sigmoid colon (25%) were the most frequent anatomical locations (Fatemeh et al., 2014). Although it was reported that standard treatments could be effective to nearly 90% of the early stage CRC patients, there were still 40 to 60% of patients at late stage or even recurring stage that had few available therapy options (Ehrig et al., 2013). To reduce this still considerably high treatment failure rate, it is necessary to find out more efficient treatment regimens.

MicroRNAs (miRNAs) are a new class of endogenous non-coding RNA molecules with a length of 19~25 nt that are cleaved from 70- to 100-nucleotide hairpin pre-miRNA precursors. A growing body of evidence indicates that, through translational repression or mRNA cleavage/decay, miRNAs could regulate the expression of nearly one-third of human genes and play important roles in cell growth, proliferation, differentiation and death (Ambros, 2004; Farh et al., 2005; Lim et al., 2005; Lu et al., 2005). Lots of miRNAs are aberrantly expressed in human cancers, including colorectal cancer (Lin et al., 2011). Furthermore, it is believed that miRNAs are associated with the development of cancers: some have an oncogenic function, while others have a tumor suppressive function (Schickel et al., 2008).

MiR-133b was initially considered to be a muscle-specific miRNA and was shown to be involved in the development of skeletal muscle (Panguluri et al., 2010; Koutsoulidou et al., 2011). However, current studies indicate a broader expression pattern of miR-133b in diverse tissues. Moreover, most of the studies showed that miR-133b was down-regulated in some human malignancies, such as osteosarcoma (Novello et al., 2013), gastrointestinal stromal tumor (Yamamoto et al., 2013), gastric cancer (Wen et al., 2013) as well as bladder cancer (Yamasaki et al., 2012). Therefore, up-regulating miR-133b or exogenously providing its analogous pharmaceutical compounds might provide effective cancer therapies for tumors.

Although Banders and his colleagues have previously

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demonstrated that miR-133b is significantly down regulated in colorectal cancer (Bandres et al., 2006), its precise role still remains unknown. In this study, we detected differential expression of miR-133b in human CRC tissue and adjacent non-tumor tissues using quantitative RT-PCR analysis and we hypothesized that miR-133b acts as a tumor suppressor. Consistent with this hypothesis, we found that overexpression of miR-133b inhibited the growth of two colorectal cancer cells, SW-620 and HT-29. Subsequently, we confirmed that TATA box-binding protein-like protein 1 (TBPL1) was direct target of miR-133b and its protein expressed higher in CRC tissue. When TBPL1 was inhibited by specific siRNA, above two cell lines also showed lower proliferation rate. It helped us to understand the tumor suppressive function of miR-133b. And, we aimed to reveal a new regulatory mechanism of miR-133b in the development of CRC, and provide a new miRNA and target gene for clinical application.

Materials and Methods

Cells and culture conditions

The human colon adenocarcinoma cells SW-620 and human colorectal adenocarcinoma cells HT-29 were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Science. The cells were maintained in Dulbecco's Modified Eagle's Medium with high glucose supplemented with 10% heat-inactivated FBS (Hyclone, United States), penicillin (100 U/mL) and streptomycin (100 mg/mL) at 37°C in a humidified atmosphere with 5% CO2. The medium was changed every 2-3 d, and the cells were trypsinized with trypsin when 80%-90% confluence was reached.

Clinical Specimen

Human CRC tissue and adjacent non-tumor tissue were obtained from patients diagnosed as colon adenocarcinoma in Department of General Surgery, The Third XiangYa Hospital of Central-South University. Stage of disease was reported according to TNM classification (Ayude et al., 2013). The specimens were obtained after surgical resection, immediately frozen at -80°C until use. The study methodologies conformed to the standards set by the Declaration of Helsinki. Collection and usage of all specimens were approved by the local Ethics Committee. For immunohistological assay, TBPL1 expression was detected by anti-TBPL1 (Santa 1:200) primary antibodies. Secondary antibody was conjugated to horseradish peroxidase (HRP).

Quantitative Real-Time PCR Analysis

To detect hsa-miR-133b level, total RNA from cultured cell or tissue was isolated using the miRNeasy Mini Kit (QIAGEN, Germany). According to the instruction of All-in-OneTM miRNA qRT-PCR Detection Kit (GeneCopoeia, MD, USA), miRNA was added with poly (A) prior to being reverse-transcribed to cDNA. This reaction was performed at 37°C for 60 min and 85°C for 5 min. U6 small nuclear RNA was used as an endogenous control in miRNA detection. The specific forward primers were purchased from GeneCopoeia (USA) and the universal reverse primers were provided in the All-in-OneTM miRNA qRT-PCR Detection Kit. Amplification was performed at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s and 72°C for 10 s. Relative quantification of target RNA expression was evaluated using the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method. Each assay was performed in triplicate and repeated at least three times to calculate mean normalized gene expression ± SE.

RT-PCR

Total RNA was prepared from the indicated cells using RNeasy Mini Kit (Qiagen) according to the manufacturer's recommendations. For the RT-PCR, first strand cDNA was generated using SuperScript II reverse transcriptase (Invitrogen) and random primers. The resultant cDNA was subjected to the PCR-based amplification. The oligonucleotide primers used in this study were as follows:human TBPL1, 5'-CCTCTTCCCACGGATGTGAT-3' (sense) and 5'- GA GTCCAATGTGCAGCAG T-3' (reverse); human GAPDH, 5'-ACCTGACCTGCCGTCTAGAA-3' (forward) and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse). The expression of GAPDH was measured as an internal control. The PCR products were subjected to 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

Transfection of miR-133b mimics

FAM-conjugated double-stranded hsa-miR-133b oligonucleotide mimics (sequence: Forward, 5'-UUUGGU CCCCUUCAACCAGCU A-3'; Reverse, 5'-GCUGGUUGAAGGGGACCA AAU U-3'), scrambled control (NC) (sequence: Forward, 5'-UUCUCCGAACGUGUCACG-3' UTT-3'; Reverse, ACGUGACACGUUCGGAGAATT-3') were designed and synthesized by GenePharma (Shanghai, China). Cells were seeded in serum containing media without antibiotics in six-well plates at the density of 4×10^6/ well approximately 24h before transfection. All cell transfections were performed with miRNA mimics or NC in antibiotic-free Opti-MEM I Reduced Serum medium with final RNA concentration at 30nM following the technical manual of Lipofectamine 2000 transfection reagent (Invitrogen). Transfection efficiency was measured 6h later by observing under fluorescence microscope and FCM assay. Other experiments were carried out 48h after transfection.

Cell proliferation assay

Cell proliferation was determined by a colorimetric method based upon metabolic reduction of the soluble yellow tetrazolium dye 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) to its insoluble purple formazan. Approximately 1×10^4 cells/ well were grown in 96-well plates and incubated overnight in 100μl of the culture medium. Cells were starved without FBS for 24 h at 70–80% confluence and then grown under indicated condition for 24 h, 48h or 72h. Each well was added with 10 μl MTT (0.5 mg/ml) and incubated for 4 h

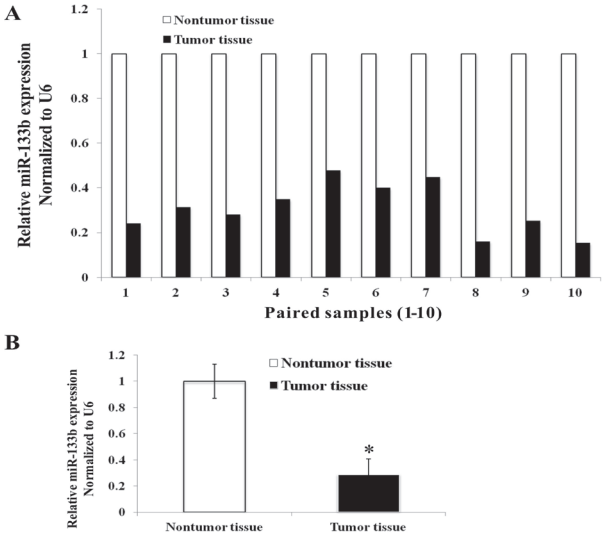

Figure 1. Expression of Hsa-miR-133b was Down Regulated in CRC. A, The expression level of miR-133b in 10 pairs of CRC tissues and matched adjacent non-tumor tissues was detected by quantitative RT-PCR. U6 snRNA was used as an endogenous control. The miR-133b levels were significantly down regulated in CRC tissues as determined by the Wilcoxon matched pairs test. B, The relative expression level of miR-133b. **P*<0.01 vs non-tumor tissue

before supernatant was removed. After plate was placed at 37 °C for 15min in 75μl dimethyl sulfoxide (DMSO), the absorbency was measured with a micro ELISA reader at a wavelength of 492 nm.

Western blotting assay

Total protein of the tissues or cultured cells was prepared using Total Protein Extraction Kit (Promab) according to the manufacturer's instruction. Protein concentrations were determined by bicinchoninic acid protein assay with bovine serum albumin as standard. The protein extracts (40 ug) were then subjected to 10% SDS–PAGE electrophoresis and subsequent nitrocellulose membrane transfer. After blocking with 5% degreased milk in Tris-buffered saline containing 0.1% Tween-20 (TBST), the membranes were incubated overnight at 4 °C with Goat TBPL1 antibody (Santa) or Mouse GAPDH antiboday (Santa). After washing 3 times with TBST at room temperature, the membranes were incubated for 1 h with corresponding horseradish peroxidase-conjugated secondary antibody. The bound antibody was detected by enhanced chemiluminescence on an X-ray film.

Transfection of luciferase reporter plasmid and Luciferase Assay

Hsa-miR-133b oligonucleotide mimics, hsa-miR-133b inhibitors (sequence: 5'-UAGCUGGUUGAAGGGGACCAAA-3'), scrambled control (NC) were designed and synthesized by GenePharma (Shanghai, China). The 1.77-kb 3' untranslated region (UTR) of human TBPL1 from a human cDNA library was amplified using PCR and cloned into a pGL3-Promoter Vector (Promega) to generate a Wt-pGL3-Promoter-TBPL1-3'UTR vector (WT vector). The following primers were used: forward:5'-CTCTCTAGACTCAAAAGAAAACTGGACCAAC-3'; and reverse: 5'- CTCTCTAGATGCACATTCAATTGAAAAA-3'. Site-directed mutagenesis of the miR-133b target sites in the TBPL1 3'-UTR was performed using mutation primers to generate the Mut-pGL3-Promoter-TBPL1-3'-UTR vector (Mut vector). The following primers were used: forward: forward: 5'-CTCTCTAGACTCAAAAGAAAACTGCAATAATTGAGGAAAT-3';andreverse:5'-CTCTCTAGATGCACATTCAATTGAAAAA-3'. After SW-620 cells were plated in a 12-well plate at ~80% confluence, the experiments were conducted into two parts. For one part, cells were co-transfected with Wt vector, and 30nM miRNA NC, or hsa-miR-133b mimics or hsa-miR-133b inhibitor. For the other part, cells were co-transfected with miR-133 mimic and pGL3-Promoter vector, WT vector or Mut Vector. Each sample was also cotransfected with 0.05 μg phRL-null plasmid expressing Renilla Luciferase (Promega) as an internal control for transfection efficiency. Cell transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Luciferase assay was performed 48 h after transfection using Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity for each transfected well. Each assay was replicated 3 times.

Suppression of TBPL1 by siRNA transfection

To knockdown TBPL1 expression, siRNA transfections were done by using HiPerFect transfection reagent (Qiagen) and four Flexitube gene solution siRNA (Qiagen) or a scrambled siRNA (control) for SW-620 and HT-29 cells according to the manufacturer's instruction.

Statistical analysis

Data are presented as the mean ± SD, and compared using Student's t-test in SPSS version 11. Double-tailed *P*-value <0.05 was considered to be statistically significant. Differences between the miR-133b in tumor tissues and adjacent non-tumor tissues were analyzed by the Wilcoxon matched pairs test.

Results

Expression of miR-133b was down-regulated in CRC tissue

To test the expression of miR-133b in human CRC tissues, we identified miR-133b levels in 10 pairs of colon adenocarcinoma samples and adjacent non-tumor tissues using quantitative RT-PCR. According to TNM classification, seven patients were in stage II and three were in stage III. Like reported by Bandres (Bandres et al., 2006), our results showed that the expressions of miR-133b were obvious lower in CRC tissue than non-tumor tissue in all ten pairs (Figure 1A). The relative expression was dropped by 71.2% (Figure 1B, *P*<0.01).

MiR-133b inhibited colon adenocarcinoma cells proliferation

In order to investigate the effect of miR-133b on colon adenocarcinoma cell proliferation, two cell lines, SW-620 and HT-29, were transfected with hsa-miR-133b mimics. As seen in Figure 2A, after transfection for 6h, the

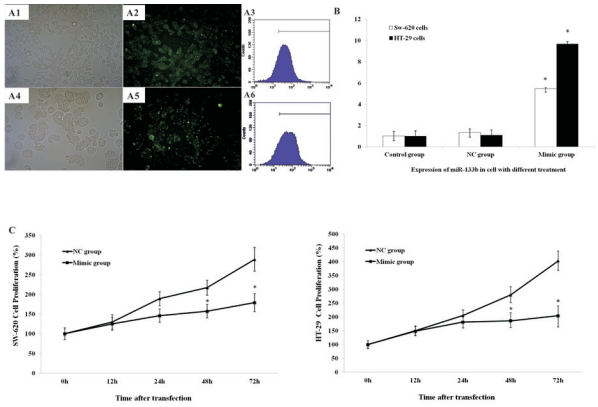

Figure 2. Overexpression of Hsa-miR-133b Inhibited Colon Adenocarcinoma Cell Proliferation. A, SW-620 and HT-29 cells transfected with FAM-conjugated hsa-miR-133b oligonucleotide mimics (mimic group) or scrambled control (NC group) for 6h exhibited green fluorescent signals under an inverted microscope (A1, A4) and an fluorescence microscope (A2, A5), respectively. FCM analysis results were shown in A3 (78%) and A6 (85%), respectively. B, Expression of miR-133b was measured by quantitative RT-PCR in control group, mimic group or NC group 48h after transfection. *$P < 0.01$ vs control group or NC group. C, The growth curves of the cells transfected with FAM-conjugated hsa-miR-133b mimics or scrambled control (NC) were compared using MTT assay. *$P < 0.01$ vs corresponding time point in NC group (Student's t test)

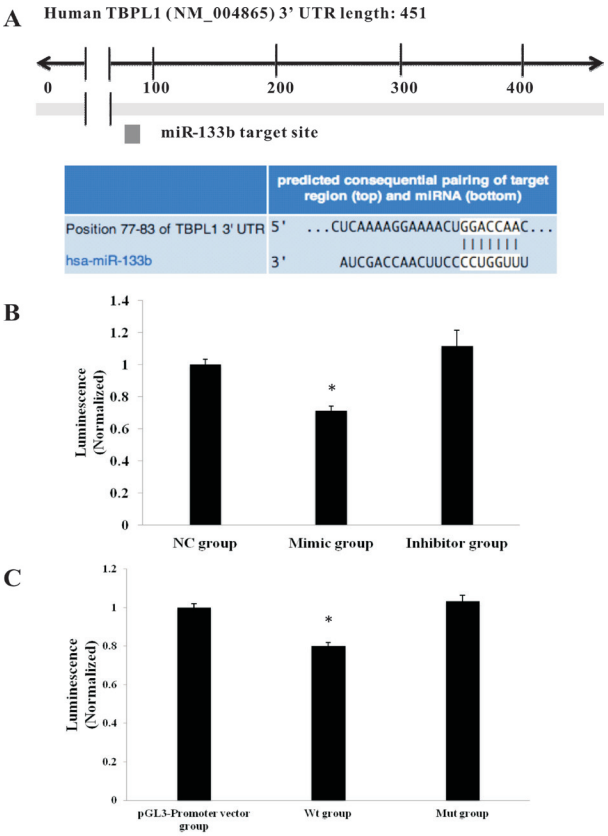

Figure 3. MiR-133b Binding Sites in 3'-UTR of TBPL1 mRNA and Luciferase Reporter Assay. A, miR-133b binding site in 3'-UTR of TBPL1 mRNA. B, Luciferase reporter assay using the vector encoding full-length of 3'-UTR of TBPL1 mRNA. The firefly luciferase values were normalized by the renilla luciferase values. *$P < 0.01$ vs NC group. C, Luciferase reporter assay using miR-133b mimics. The firefly luciferase values were normalized by the renilla luciferase values. *$P <$ 0.01 vs pGL3-Promoter vector group

transfection efficiency, determined by green fluorescence in cytoplasma and FCM analysis, was approximately 78% and 85% for SW-620 and HT-29 cells, respectively. And then, we surveyed the basal expression of miR-133b in cells. As expected, the basal expression in both SW-620 and HT-29 cells was only at detection limit, which was too low to be seen in Figure 2B. After transfection of miR-133b mimics, its expression level had been obviously enhanced for at least 48h.

Further, MTT assay was performed to detect cell proliferation. As shown in Figure 2C, transfection of miR-133b dramatically suppressed cell proliferation. It was revealed that, compared with that in NC group, the cell growth in mimic group begun to slow down at 24h and had been lasted till 72h after transfection. The differences were significantly obvious at 48h and 72h points between mimic group and NC group (P<0.01).

TBPL1 is a target gene of miR-133b

The sequences of miR-133b were confirmed by referring to miRBase (release 20.0, June 2013; http://microrna. sanger.ac.uk/). When predicted using PICTAR, it indicated that there are 400 predicted genes targeted by miR-133b that includes TBPL1. According to the sequences listed in http://www.targetscan.org, the 3'-UTR of TBPL1 mRNA (NM_004865) contains one putative binding site of miR-133b (Figure 3A).

To explore if this site is responsible for the negative regulation by miR-133b, we integrated the full length of TBPL1 3'-UTR or a fragment with mutated target sites in TBPL1 3'UTR into a luciferase reporter vector and tested the effect of miR-133b on luciferase activity in SW-620 cells. The results showed that the luminescence intensity was significantly reduced in the miR-133b mimics transfected cells, but not in the miR-133b inhibitors transfected cells, suggesting that TPBL1 had actual binding site of miR-133b (P<0.01, Figure 3B). When mutant TBPL1 3'UTR was transfected, luminescence intensity had no difference with that in cells transfected with pGL3-Promoter vector alone (P>0.05, Figure 3C).

At the same time, when SW-620 and HT-29 were transfected with miR-133b, TBPL-1 protein level was obvious lower than that in control cells or cells transfected with NC (Figure 4C).

Expression of TBPL1 in CRC tumor was higher than normal

Since it was confirmed that TBPL1 could be targeted by miR-133b, which was down regulated in both SW-620, HT-29 cells and CRC tissue, we further checked the expression of TBPL1 protein in CRC patients. As shown in Figure 4A, its expression was obvious higher in CRC tissue than non-tumor tissue in all ten pairs. Similar difference was also observed in the immunohistochemistry assay (Figure 4B).

Down-regulation of TBPL1 inhibited colon adenocarcinoma cells proliferation

At the last step, to confirm the function of TBPL1 in

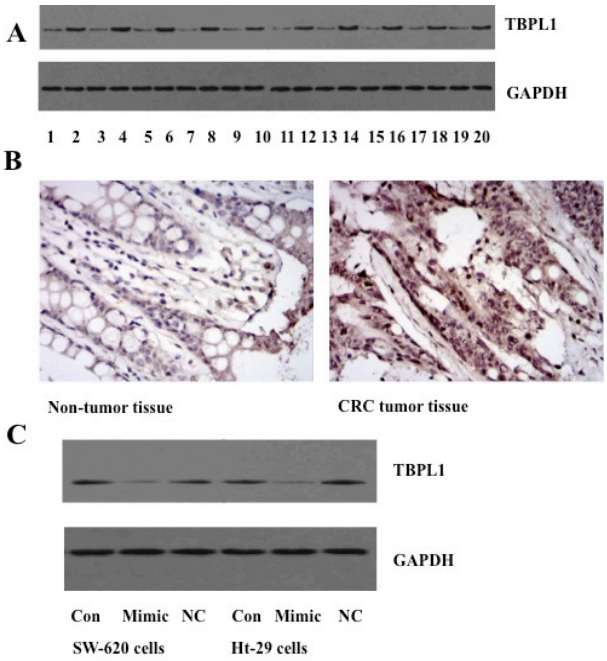

Figure 4. The Expression of TBPL1 in CRC Tissue and Colon Adenocarcinoma Cells. A, The expression level of TBPL1 in 10 pairs of CRC tissues and matched adjacent non-tumor tissues was detected by western blot. GAPDH was used as an internal control. 1, 3…17 and 19 mean tumor tissue. 2,4…18 and 20 mean matched adjacent non-tumor tissue. B, The expression level of TBPL1 in CRC tissues and adjacent non-tumor tissues measured by immunohistochemistry assay. (Original magnification, ×200). C, The expression level of TBPL1 in SW-620 and HT-29 cells in control group, miR-133b transfected group and NC transfected group, respectively. GAPDH served as internal control

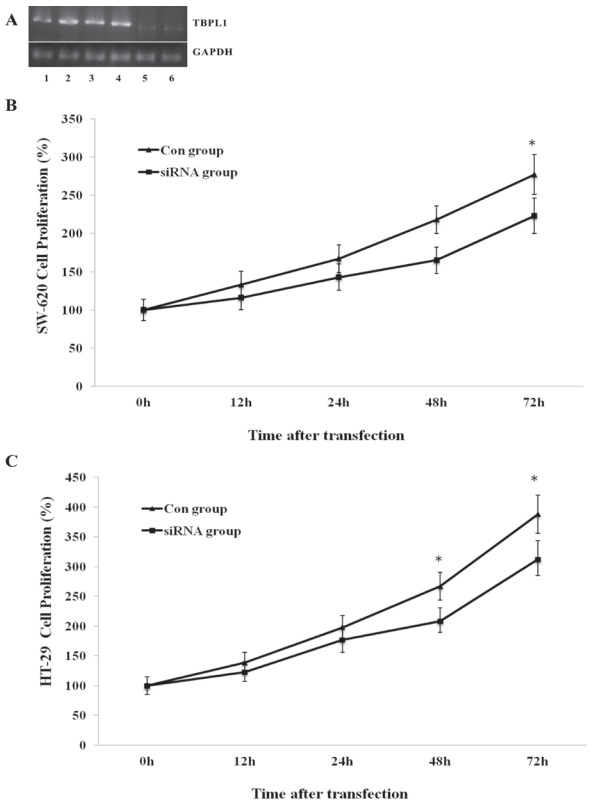

Figure 5. Cell Proliferation was Inhibited after TBPL1 siRNA Transfection. A, The expression level of TBPL1 in SW-620 and HT-29 cells transfected with TBPL1 siRNA was detected by RT-PCR. GAPGH was served as internal control. B, The growth curves of the cells were compared using MTT assay. *$P < 0.05$ vs corresponding time point in control group (Student's t test)

cell proliferation, we transfected SW-620 and HT-29 with TBPL1-specific siRNA oligonucleotides and a scrambled siRNA (control). MTT assay was conducted to measure the cell proliferation till 72 h post-transfection. As we could see in Figure 5B and C, both cells exhibited a lower proliferation rate after siRNA transfection.

Discussion

MiRNAs are endogenous non-coding RNAs that interacting with the 3'UTR of target mRNA which can induce mRNA cleavage when pairing is complete or inhibit protein synthesis repression when pairing is incomplete. In literature, there are approximately 1,000 miRNA molecules per cell, with some cells exceeding 50,000 molecules (Lim et al., 2003). Recent studied called that miRNA as 'oncomirs' because that they function either as tumor suppressors or as oncogenes (Esquela-Kerscher et al., 2006). Investigation of the differentially expressed miRNAs in cancer specimens has yielded important information on its carcinogenesis.

MiR-133b is located on chromosome 18 in the same bicistronic unit with miR-133a (Zhou et al., 2013). It has long been recognized as a muscle specific miRNA that may regulate myoblast differentiation and participate in many myogenic diseases (Koutsoulidou et al., 2011; Panguluri et al., 2010). Banders and his colleagues have previously demonstrated that miR-133b is significantly down regulated in colorectal cancer (Bandres et al., 2006). In the current study, by using real-time PCR, the lower expression of miR-133b in CRC had been confirmed. We also found that cells transfected with miR-133b mimics inhibited the cell proliferation, which strongly suggests that miR-133b acts as tumor suppressor in CRC. At the same time, similar results had also been observed in osteosarcoma (Novello et al., 2013), gastrointestinal stromal tumor (Yamamoto et al., 2013), gastric cancer (Wen et al., 2013) as well as bladder cancer (Yamasaki et al., 2012). However, the targets of miR-133b that regulates in CRC have not been established previously.

As we know, RNA polymerase II transcription initiation in eukaryotes requires the formation of a multiprotein complex around the mRNA start site. Recent studies of the three eukaryotic transcription machineries revealed that all initiation complexes share a conserved core that consists of the RNA polymerase, TBP, and some transcription factors (Vannini et al., 2012). The function of TBP in several complexes is involved in core promoter recognition and assembly of the pre-initiation complex. TBPL1, also called as TBP-like factor (TLF), is a more distant paralog of TBP that is found in all metazoans (Martianov et al., 2002). TBPL1 has only ~40% identity with the TBP core domain (Dantonel et al., 1999). It is the only member of TBP family that lacks the ability to bind the TATA box. It however, interacts with TFIIA and TFIIB.

The function of TBPL1 has been studied in worm,

frog and fish using knockdown and dominant negative strategies. Most of the studies focused on the embryonic development. It was indicated that ablation of TBPL1 function results in an early arrest of embryonic development and down regulation of a subset of genes. TBPL1 knockdown combined with transcriptome profiling in frog embryos showed that a large number of transcripts require TBPL1 but not TBP (Jacobi et al., 2007). In our study, for the first time, it was shown that TBPL1 was a target for miR-133b and expressed at lower level in CRC. We also directly confirmed that inhibition of TBPL1 by siRNA obviously slowed the cell proliferation. This indicated that TBPL1 might, at partially, be involved in the tumor suppressive role taken by miR-133b.

According to the clinical investigation, surgical resection combined with postsurgical chemotherapeutic agents remains the preferred treatment strategy for CRC patients; however, cases with above therapy still show recurrence rates between 40-60% in the first three years (Aghili et al., 2010). The results in our study indicated that miR-133b might play a suppressive role in CRC development. And, it is the first time to reveal a novel to TBPL1 being a target for miR-133b in CRC. This might be used as a potential biomarker or target for CRC diagnosis and chemotherapy.

Acknowledgements

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