Original Paper

The Epigenetically-Regulated microRNA-378a Targets TGF- β 2 in TGF- β 1-Treated Hepatic Stellate Cells

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Key Words

MicroRNA-378a • Hepatic stellate cell • TGF- β 2 • DNA methylation

Abstract

Background/Aims: In liver fibrosis, the activation of hepatic stellate cells (HSCs) is considered as a pivotal event. It is well known that transforming growth factor- β 1 (TGF- β 1) is the main stimuli factor responsible for HSC activation. microRNAs (miRNAs), regulating various biological processes, have recently been shown to be involved in HSC activation. A recent study reported that deficiency of miR-378a contributes to cardiac fibrosis via TGF- β 1-dependent paracrine mechanism. However, the involvement of miR-378a and its roles in TGF- β 1-induced HSC activation remains largely unknown. **Methods:** miR-378a expression was detected in TGF- β 1-treated cells and patients with cirrhosis. Then, effects of miR-378a overexpression on cell proliferation and HSC activation were analyzed. We also analyzed the binding of miR-378a to the 3'-untranslated region of TGF- β 2. **Results:** In response to TGF- β 1, miR-378a expression was down-regulated in a dose-dependent manner. miR-378a overexpression suppressed both cell proliferation and cell cycle in TGF- β 1-treated LX-2 cells. Moreover, miR-378a overexpression inhibited TGF- β 1-induced HSC activation including the reduction of α -smooth muscle actin (α -SMA) and type I collagen. Similarly, miR-378a resulted in a reduction in cell proliferation, and the expressions of α -SMA and Col1A1 in TGF- β 1-treated primary HSCs. Notably, TGF- β 2 was confirmed as a target of miR-378a by luciferase reporter assays. Interestingly, miR-378a promoter methylation may be responsible for miR-378a down-regulation in TGF- β 1-treated LX-2 cells and TGF- β 1-treated primary HSCs. Further studies confirmed that reduced miR-378a was associated with promoter methylation in patients with cirrhosis compared with healthy controls. **Conclusion:** Our results demonstrate that miR-378a expression is associated with

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its methylation status in TGF- β 1-treated cells, and epigenetically-regulated miR-378a inhibits TGF- β 1-induced HSC activation, at least in part, via TGF- β 2.

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Introduction

Cirrhosis, a major cause of morbidity and mortality worldwide, can be caused by a number of conditions, including sustained excessive alcohol consumption, chronic viral hepatitis, fatty liver disease and hereditary metabolic disorders [1-4]. Cirrhosis is characterized by permanent scarring of the liver that remodels liver tissues and destroys its normal functions. Liver fibrosis is the intermediate link in the progression from chronic liver disease to cirrhosis [5]. Therefore, effectively inhibiting or delaying the development of liver fibrosis contributes to reduce the risk of cirrhosis in patients with chronic liver diseases. During liver fibrosis, hepatic stellate cells (HSCs), which are mainly responsible for the accumulation of extracellular matrix (ECM) proteins, become activated and undergo myofibroblastic transdifferentiation [6-8]. Transforming growth factor- β 1 (TGF- β 1), the main profibrogenic mediator, can trigger HSC transdifferentiation via TGF- β /Smads pathway [9]. However, to date, many regulatory mechanisms of liver fibrosis are still unclear.

MicroRNAs (miRNAs) are endogenous small (~22nt) non-coding RNAs that are involved in various biological processes, including cell proliferation, apoptosis, metabolism, differentiation, immune response and development [10, 11]. miRNAs are largely known to base pair with the 3'-untranslated region (3'-UTR) of target mRNAs, repressing translation or enhancing mRNA cleavage to control gene expression [12]. Increasing evidence shows that miRNA dysregulation is found in liver fibrosis, and miRNAs play a vital role in the progression of liver fibrosis [13-15]. For example, miR-29b reverses activated HSCs to a quiescent state [16]. We previously demonstrated that miR-17-5p activates HSC via targeting Smad7 [15]. Therefore, miRNAs may act as HSC regulators in liver fibrosis.

miR-378a, down-regulated in a variety of diseases including cancers, has been reported to trigger various cellular functions such as cell growth, cell cycle and migration [17-21]. Recently, Nagalingam *et al.* found that miR-378a inhibits cardiac fibrosis via TGF- β 1-dependent paracrine mechanism [22]. However, whether miR-378a inhibits TGF β /Smads pathway in activated HSCs during liver fibrosis is largely unknown. In this study, we aimed to explore the role of miR-378a in TGF- β 1-treated cells. In addition, we investigated whether miR-378a promoter methylation was responsible for miR-378a down-regulation in TGF- β 1-treated cells.

Materials and Methods

Human specimens

Written informed consent was received from all patients prior to liver tissues. In this study, 15 healthy controls and 15 liver cirrhosis patients undergoing partial liver resection or liver biopsy were selected from the First Affiliated Hospital of Wenzhou Medical University. In addition, liver samples were obtained from healthy controls undergoing liver biopsy. Liver cirrhosis was diagnosed by liver biopsy and/or a typical appearance of the liver on abdominal ultrasound and/or computed tomography scan. This study was performed in compliance with the Declaration of Helsinki and approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University.

Carbon tetrachloride (CCl₄) liver injury model

Adult male Sprague-Dawley (180-220 g) rats were provided by the Experimental Animal Center of Wenzhou Medical University. Liver fibrosis was induced by administration of 2 ml of CCl₄/olive oil (1:1, v/v)/kg body weight by intraperitoneal injection twice weekly for up to 6 weeks [23]. Twenty rats were randomly divided into two groups. As following, rats in group 1 (n = 10) received twice weekly injections of olive oil (vehicle control) and rats in group 2 (n = 10) received twice weekly injections of CCl₄ (CCl₄-treated

rats). The animal experimental protocol was approved by the University Animal Care and Use Committee. Rats were sacrificed after the last CCl₄ injection, and liver tissues were harvested for the further analysis. Liver fibrosis in rats was confirmed by hematoxylin & eosin and Masson staining.

Cell culture and transfection

The human LX-2 cell strain was obtained from JENNIO Biological Technology (Guangdong, China). Cells were cultured in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin G sodium salt and 100 U/ml streptomycin sulfate (Gibco, Carlsbad, CA, USA), and incubated at 37°C under an atmosphere of 5% CO₂. Exponentially growing cells were treated with 0, 0.5, 2 and 5 ng/ml TGF- β 1 (R&D Systems, Shanghai, China) for 24 h. For transfection, TGF- β 1-treated cells were transfected with 10 nM miR-378a mimics (GenePharma biotechnology, Shanghai, China) using Lipofectamine 2000 (Invitrogen, USA). In addition, TGF- β 1-treated cells were also treated with 2.5 μ M 5-Aza-2'-deoxycytidine (5-Aza) (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Cells were harvested for RNA/miRNA isolation, and whole cell extracts were subjected to western blot analysis.

Isolation and culture of rat HSCs

Adult male Sprague-Dawley rats (body weight, 400-500 g) were used for HSC isolation as described previously [24]. The primary HSCs were studied at day 1 after isolation throughout all the studies. The purity of primary HSCs isolated from healthy rats was confirmed by immunocytochemical staining for α -SMA and the purity reached > 98%.

Proliferation and apoptosis assays

Cell proliferation was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium Bromide (MTT) assay in keeping with instructions of the MTT cell proliferation assay kit (Beyotime Biotechnology, Jiangsu, China). Optical density (OD) was measured at 490 nm on a microplate reader (Bio-Rad 550, USA). The activity of Caspase3 was determined using the Caspase3 activity kit (Beyotime Biotechnology). Cell lysates were prepared after the transfection of miR-378a. Then, cells were scraped, centrifuged, resuspended and lysed in a lysis buffer in accordance with the manufacturer's instructions. Assays were performed on 96-well microtitre plates by incubating 10 μ L protein of cell lysate per sample in 80 μ L of reaction buffer (1% NP-40, 20 mM Tris-HCl, pH 7.5, 137 mM NaCl and 10% glycerol) containing 10 μ L of 2 mM Caspase3 substrate (Ac-DEVD-pNA). Lysates were incubated at 37 °C for 4 h. Samples were measured using a microplate reader (Bio-Rad) at A₄₀₅ in accordance with the manufacturer's instructions.

Cell cycle analysis

The Cell Cycle Analysis Kit (Beyotime, China) was used to analyze cell cycle. Cells were fixed in 70% ethanol in PBS at -20°C for 24 h and then labeled with 0.5 ml propidium iodide (PI) staining buffer containing 200 mg/ml RNase A and 50 μ g/ml PI at 37°C for 40 min in the dark. Analyses were performed on a BD LSR flow cytometer (BD Biosciences).

Quantitative real-time PCR (qRT-PCR)

To examine mRNA expression, total RNA was extracted from liver tissues and cells using TRIzol Kit (Life Technologies). Equal amounts of total RNA were reverse-transcribed to cDNA using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). Gene expression was measured by real-time PCR using SYBR Green real-time PCR Master Mix (Toyobo, Osaka, Japan). The primers of α -1 (I) collagen (Col1A1), α -SMA, GAPDH, U6, DNA methyltransferase 1 (DNMT1), DNMT3a and DNMT3b were designed as described previously [5, 13]. The primers used for TGF- β 2 were 5'-AGAGCCTTGTTTCATCAGTGT-3' and 5'-TGCTTCTGTCTCTCTCACTTACA-3'. To examine miRNA expression, total RNA was extracted from liver tissues and cells using a miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). miR-378a expression was detected using the TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA). The GAPDH and U6 snRNA levels were used to normalize the relative abundance of mRNAs and miRNAs, respectively.

Western blot analysis

The BCA protein assay kit (Beyotime Biotechnology, Jiangsu, China) was used to assess sample protein concentration. Antibodies against DNMT1, DNMT3a, DNMT3b, TGF- β 2, type I collagen, α -SMA and GAPDH

were obtained from Abcam (Cambridge, MA, USA). Proteins (30–50 μ g) were separated by SDS-PAGE and then transferred to Immobilon P membranes (Millipore, Bedford, MA, USA). After blocking, the membranes were incubated with primary antibodies overnight at 4°C and then with secondary antibodies at 37°C for 1 h. The antigen-antibody complex was developed by enhanced chemiluminescence, exposed in the dark room and analyzed for integral absorbance (IA) of the protein bands using quantitative software, Quantity One 4.4.

Methylation analysis

miR-378a CpG island was searched in UCSC Genome Browser. About 0.5 μ g genomic DNA was treated with sodium bisulphite and subjected to PCR. The hsa-miR-378a primers for PCR were 5'-GGCCCCGTGCAGACGC-3' and 5'-TATCGGCCTCTGAGCAAGTCC-3'. The rno-miR-378a primers for PCR were 5'-GGAGGATCTGCTGGACCGAG-3' and 5'-GGAAGAGACGAGAAGGTCC G-3'. The bisulphite-sequencing analysis was carried out as described previously [25].

Luciferase activity assay

LX-2 cells were co-transfected with either luciferase reporter plasmid harboring wild-type TGF- β 2 3'UTR (pmirGLO-TGF- β 2-wt) or mutant TGF- β 2 3'UTR (pmirGLO-TGF- β 2-mut) together with miR-378a mimics or miR-NC. The 3'UTR of TGF- β 2 was cloned downstream of the firefly luciferase gene. If the interaction between miR-378a and the 3'UTR of TGF- β 2 mRNA was existed, the activity of firefly luciferase was decreased. Approximately 48 h after transfection, the cells were harvested and luciferase activity was determined by a luminometer using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical analysis

Data from at least three independent experiments were expressed as the mean $\pm$ SD. Comparisons between two groups and multiple groups were made using Student's *t*-test and one-way analysis of variance, respectively. *P* < 0.05 was considered significant. All statistical analyses were performed with SPSS software (version 13; SPSS, Chicago, IL).

Results

miR-378a is down-regulated in TGF- β 1-treated HSCs

Exposure of HSC to TGF- β 1, the master fibrogenic cytokine stimulating HSC activation, resulted in the transformation of HSC to myofibroblasts [26]. α -SMA, a key marker widely accepted for HSC activation, is often expressed at high levels in activated HSCs [27]. In our experiments, the human HSC line, LX-2, was treated with different concentrations of TGF- β 1, and the effects on α -SMA and miR-378a expression were examined. TGF- β 1 induced an increase in α -SMA expression in a dose-dependent manner, ranging from concentrations of 0.5 to 5 ng/ml (Fig. 1A). By contrast, there was a significant decrease in miR-378a expression

Fig. 1. Effects of TGF- β 1 on the expressions of α -SMA and miR-378a in LX-2 cells. The LX-2 cells were treated with TGF- β 1 (0, 0.5, 2 and 5 ng/ml) for 24 h. (A) α -SMA expression was dose-dependently induced by TGF- β 1. (B) miR-378a expression was dose-dependently down-regulated by TGF- β 1. Each value is the mean $\pm$ SD of three experiments. **P* < 0.05 compared with the control.

Fig. 2. Effects of miR-378a overexpression on cell proliferation, apoptosis, cell cycle, HSC transdifferentiation and collagen expression. LX-2 cells were treated with TGF- β 1 (2 ng/ml) for 24 h and then transfected with 10 nM miR-378a mimics for 24 h. (A) TGF- β 1-induced cell proliferation was inhibited by miR-378a. Data were analyzed using the MTT assay. Effects of miR-378a on cell apoptosis (B) and cell cycle (C) in TGF- β 1-treated cells. Cell apoptosis was determined by Caspase3 activity assay. Effects of miR-378a on TGF- β 1-induced α -SMA mRNA (D) and protein (E) expression. Effects of miR-378a on TGF- β 1-induced Col1A1 mRNA (F) and protein (G) expression. Each value is the mean $\pm$ SD of three experiments. * P <0.05 compared with the control and # P <0.05 compared with TGF- β 1 group.

as we increased the concentrations of TGF- β 1 from 0.5 to 5 ng/ml, indicating that the inhibitory effect of TGF- β 1 on miR-378a was also dose-dependent (Fig. 1B). Taken together, our data suggest that miR-378a may play a role in TGF- β 1-mediated liver fibrosis.

miR-378a suppresses cell proliferation, cell cycle and HSC activation

Next, we investigated whether miR-378a overexpression suppressed TGF- β 1-induced cell proliferation. As indicated by the results of MTT assays, miR-378a significantly inhibited

Fig. 3. Effects of miR-378a on cell proliferation, α -SMA and Col1A1. Primary 1-day-old HSCs were treated with TGF- β 1 (2 ng/ml) for 24 h and then transfected with 10 nM miR-378a mimics for 24 h. (A) Cell proliferation enhanced by TGF- β 1 was suppressed by miR-378a. Data were analyzed using the MTT assay. (B) The mRNA expressions of α -SMA and Col1A1 induced by TGF- β 1 were inhibited miR-378a. Each value is the mean $\pm$ SD of three experiments. * P <0.05.

Fig. 4. Interaction of miR-378a with the 3'UTR of TGF- β 2 in HSCs. LX-2 cells or primary 1-day-old HSCs were treated with TGF- β 1 (2 ng/ml) for 24 h and then transfected with 10 nM miR-378a mimics for 24 h. Effects of miR-378a on TGF- β 2 mRNA (A) and protein expression (B) in TGF- β 1-treated LX-2 cells. (C) Effects of miR-378a on TGF- β 2 protein in TGF- β 1-treated primary HSCs. (D) miR-378a-binding sites in the 3'UTR of TGF- β 2 mRNA based on microRNA.org software. (E) Analysis of luciferase activity in cells transfected with pmirGLO-TGF- β 2-wt or pmirGLO-TGF- β 2-mut as well as those co-transfected with miR-378a mimics or miR-NC (n = 3). Each value is the mean $\pm$ SD of three experiments. * P <0.05 compared with the control.

TGF- β 1-induced cell proliferation (Fig. 2A). Further study showed that no significant changes in caspase3 activity were found in TGF- β 1-treated cells after miR-378a mimics transfection, suggesting that cell apoptosis was not affected by miR-378a in TGF- β 1-treated cells (Fig. 2B). Notably, miR-378a suppressed cell cycle in TGF- β 1-treated cells, with an increase in the number of cells in the G0/G1 phase and a reduction in the number cells in the S phase (Fig. 2C). Then, whether miR-378a overexpression has an effect on HSC transdifferentiation

Fig. 5. The promoter methylation status of miR-378a *in vitro* and *in vivo*. (A) Schematic representation of CpG sites in the promoter region of hsa-miR-378a. Each vertical bar represents the presence of a CpG dinucleotide. (B) The methylation of miR-378a was detected by bisulfite sequencing in LX-2 cells treated with TGF- β 1 (0, 0.5, 2 and 5 ng/ml). Each row of boxes represents the sequence analysis of an individual clone from the PCR products (white box, unmethylated CpG site; black box, methylated CpG site). (C) Schematic representation of CpG sites in the promoter region of rno-miR-378a. (D) The methylation of miR-378a was detected by bisulfite sequencing in primary HSCs. Primary 1-day-old HSCs were treated with TGF- β 1 (2 ng/ml) for 24 h and then transfected with miR-378a (10 nM) mimics or 5-Aza (2.5 μ M) for 24 h. (E) miR-378a expression in TGF- β 1-treated cells after 5-Aza treatment. (F) The methylation of miR-378a was detected by bisulfite sequencing in primary HSCs isolated from healthy rats or CCl₄-treated rats. (G) miR-378a expression in primary HSCs isolated from healthy rats or CCl₄-treated rats. Each value is the mean $\pm$ SD of three experiments. * P <0.05.

was examined. The results of qRT-PCR showed that miR-378a significantly inhibited TGF- β 1-mediated α -SMA mRNA expression (Fig. 2D). In line with it, western blot analysis demonstrated that miR-378a inhibited TGF- β 1-induced α -SMA protein expression (Fig. 2E). To further confirm the role of miR-378a in HSC activation, the effects of miR-378a on collagen expression were examined. As expected, the mRNA and protein levels of Col1A1 induced by TGF- β 1 were suppressed by miR-378a (Fig. 2F and Fig. 2G). The effects of miR-378a overexpression on HSC activation were also examined in primary HSCs isolated from healthy rats. It was found that cell proliferation, and the mRNA expressions of α -SMA and Col1A1 were inhibited by miR-378a in TGF- β 1-treated primary HSCs (Fig. 3). All these results suggest that HSC activation could be suppressed by miR-378a overexpression.

TGF- β 2 is a target of miR-378a

To further determine the underlying mechanism of the inhibitory role of miR-378a in HSC activation, bioinformatic analysis (microRNA.org) was employed to identify the potential targets of miR-378a. Among the miR-378a target genes, we focused on TGF- β 2 due to its role in TGF- β /Smad signaling. To explore the functional role of miR-378a in TGF- β 2 expression, TGF- β 2 expression was examined in TGF- β 1-treated LX-2 cells transfected with miR-378a mimics. Interestingly, the mRNA and protein levels of TGF- β 2 were inhibited by miR-378a in TGF- β 1-treated cells (Fig. 4A and Fig. 4B). The similar results were also seen in primary HSCs. It was found that miR-378a caused a reduction in TGF- β 2 protein expression in TGF- β 1-treated primary HSCs (Fig. 4C). To understand by what mechanism miR-378a decreased levels of TGF- β 2, we further investigated whether TGF- β 2 was the direct target of miR-378a. Next, the 3'UTR target sequence of TGF- β 2 mRNA was cloned into the pmirGLO plasmid to confirm whether miR-378a could directly regulate TGF- β 2 expression via the predicted binding site (Fig. 4D). miR-378a significantly reduced luciferase activities of pmirGLO-TGF-

Fig. 6. The expression and methylation status of miR-378a in TGF- β 1-treated LX-2 cells after 5-Aza treatment. (A) The methylation of miR-378a in TGF- β 1-treated LX-2 cells after 5-Aza treatment. Each row of boxes represents the sequence analysis of an individual clone from the PCR products (white box, unmethylated CpG site; black box, methylated CpG site). (B) miR-378a expression in TGF- β 1-treated LX-2 cells after 5-Aza treatment. Each value is the mean $\pm$ SD of three experiments. * P <0.05 compared with the control and # P <0.05 compared with TGF- β 1 group.

Fig. 7. The expression and methylation status of miR-378a in patients with cirrhosis. (A) miR-378a expression was significantly lower in patients with cirrhosis. (B) The methylation status of miR-378a in patients with cirrhosis. Each row of boxes represents the sequence analysis of an individual clone from the PCR products (white box, unmethylated CpG site; black box, methylated CpG site). (C) The mean frequency of methylation was significantly higher in patients with cirrhosis. Each value is the mean $\pm$ SD of three experiments. * P <0.05 compared with healthy controls.

β 2-wt, whereas miR-378a had no effect on luciferase activities of pmirGLO-TGF- β 2-mut (Fig. 4E). Our data suggest that TGF- β 2 is a target of miR-378a.

Promoter methylation status and miR-378a expression in TGF- β 1-treated HSCs after 5-Aza demethylation

A number of studies have indicated that aberrant methylation may be responsible for miRNAs down-regulation in some malignant cells [28-30]. Next, we explored whether

Fig. 8. Expressions of DNMT1, DNMT3a and DNMT3b in TGF- β 1-treated LX-2 cells. LX-2 cells were treated with TGF- β 1 (2 ng/ml) for 24 h. (A) The mRNA expressions of DNMT1, DNMT3a and DNMT3b were enhanced in cells after TGF- β 1 treatment. (B) The protein expressions of DNMT1, DNMT3a and DNMT3b were enhanced in cells after TGF- β 1 treatment. Each value is the mean $\pm$ SD of three experiments. * P <0.05.

the promoter of miR-378a was aberrantly methylated in TGF- β 1-activated HSCs. The methylation status of the CpG island located hsa-miR-378a promoter was detected by bisulfite-sequencing analysis (Fig. 5A). The mean frequency of miR-378a methylation was 23.2% in LX-2 cells without TGF- β 1 (Fig. 5B). Then, LX-2 cells were treated with different concentrations of TGF- β 1. Compared with the control, there was a significant increase in methylation frequency in TGF- β 1-treated cells, with the highest methylation frequency at the concentration of 5 ng/ml (Fig. 5B). As shown in Fig. 1B, miR-378a was reduced in TGF- β 1-treated cells in a dose-dependent manner. Therefore, there may be a negative correlation between miR-378a expression and miR-378a methylation. Using bioinformatic analysis, there was also a CpG island in the rno-miR-378a promoter (Fig. 5C). Then, the methylation status of the CpG island located rno-miR-378a promoter was assessed in primary HSCs. We found that compared with Day 1, miR-378a methylation was enhanced in TGF- β 1 group (Fig. 5D). By contrast, miR-378a expression was reduced in TGF- β 1 group compared with Day 1 (Fig. 5E). In line with these, miR-378a methylation was increased in primary HSCs isolated from CCl₄ rats relative to healthy rats (Fig. 5F). Similarly, miR-378a expression was reduced in primary HSCs isolated from CCl₄ rats relative to healthy rats (Fig. 5G). To further confirm whether miR-378a expression is associated with promoter methylation, cells were treated with DNMT inhibitor 5-Aza. We found that the mean frequency of miR-378a methylation was 63.2% in cells treated with TGF- β 1, which was reduced to 36.8% in TGF- β 1-treated cells after 5-Aza treatment (Fig. 6A). Consistent with the results of methylation, miR-378a expression down-regulated by TGF- β 1 was restored by 5-Aza (Fig. 6B). Likewise, 5-Aza reduced miR-378a methylation and enhanced miR-378a expression in TGF- β 1-treated primary HSCs (Fig. 5D and Fig. 5E). These data suggest that miR-378a expression is associated with promoter methylation during liver fibrosis.

Promoter methylation status and miR-378a expression in patients with liver cirrhosis

To further confirm the relation between miR-378a expression and promoter methylation, miR-378a methylation and expression were detected in 15 healthy controls and 15 patients with cirrhosis. It was found that miR-378a was down-regulated in patients with cirrhosis, compared with healthy controls (Fig. 7A). By contrast, compared with healthy controls, the mean frequency of miR-378a methylation was enhanced in patients with cirrhosis (Fig. 7B and Fig. 7C).

DNMTs are involved in the effects of TGF- β 1 on miR-378a methylation

Next, to further determine whether DNMTs are involved in the effects of TGF- β 1 on miR-378a methylation, the mRNA and protein levels of DNMTs including DNMT1, DNMT3a and DNMT3b were examined in TGF- β 1-treated LX-2 cells. As expected, the mRNA and protein levels of DNMT1, DNMT3a and DNMT3b were enhanced in TGF- β 1 group relative

to the control, suggesting that DNMTs are involved in the effects of TGF- β 1 on miR-378a methylation (Fig. 8).

Discussion

Increasing evidence suggests that miR-378a plays a vital role in a variety of diseases [31, 32]. In colorectal cancer, miR-378a inhibits colorectal cancer cell growth and colony formation, and induces cell cycle arrest [19]. In breast cancer, miR-378a modulates tamoxifen sensitivity in MCF-7 cells through targeting GOLT1A [18]. In organ fibrosis, miR-378a is a negative regulator of TGF- β 1 and down-regulation of miR-378a contributes to the development of cardiac fibrosis [22]. In inflammation, miR-378a is found to be reduced in activated NK cells and targets granzyme B. IFN- α activation decreases miR-378a expression and in turn augments NK cell cytotoxicity [33]. In this study, we found that miR-378a was reduced in TGF- β 1-activated HSCs, which was consistent with the previous reports in cancers [17, 19]. miR-378a suppressed cell proliferation and cell cycle in TGF- β 1-treated LX-2 cells, with no changes in cell apoptosis. In addition, miR-378a caused the inhibition of HSC activation, with a reduction in α -SMA and Col1A1. The similar results were also seen in primary HSCs. miR-378a overexpression resulted in a reduction in cell proliferation, and the expressions of α -SMA and Col1A1 in primary HSCs treated with TGF- β 1. All the data suggest an inhibitory role of miR-378a in liver fibrosis.

Aberrant activated TGF- β /Smad signal pathway has been reported to contribute to the activation of HSC and the development of liver fibrosis [34-36]. In the liver, the TGF- β family contains three closely related isoforms (i.e., TGF- β 1, TGF- β 2, and TGF- β 3), which have similar biological activities in liver fibrosis [37]. TGF- β 1, as the main profibrogenic mediator, mainly activates HSC and causes liver fibrosis through TGF- β 1/Smad signal pathway [35, 38]. Many studies showed that TGF- β 2 can induce fibrotic marker expression and play a vital role in the fibrotic diseases [39]. For example, TGF- β 2 contributes to cell proliferation and α -SMA expression in myofibroblasts [40]. Recently, TGF- β 2 has been demonstrated to have a pro-fibrotic role in liver and kidney fibrosis [41, 42]. Further study showed that TGF- β 2 can be induced by TGF- β 1 in HSCs [43]. In this study, the mRNA and protein expressions of TGF- β 2 were inhibited by miR-378a in TGF- β 1-treated cells. Further study showed that TGF- β 2 was confirmed as a direct target of miR-378a. Taken together, miR-378a inhibited TGF- β 1-induced HSC activation, at least in part, via TGF- β 2. However, there may be still other signaling pathways involved in miR-378a regulating HSCs function. Therefore, the underlying mechanism and function of miR-378a in HSC may be more complex than we imagine, further functional analysis will be needed in the future.

DNA methylation, a type of epigenetic modifications in mammals, plays a crucial role in normal transcriptional regulation. For example, hypermethylation of the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is associated with loss of PTEN expression during liver fibrosis. Previously, we reported that curcumin inhibits HSC activation via PTEN demethylation [13]. Interestingly, methylation is also involved in miRNA expression. In K-562 cells, epigenetic modification may be responsible for the deregulation of miR-663 expression [28]. In this study, miR-378a expression was reduced by TGF- β 1 in a dose-dependent manner. Moreover, miR-378a methylation was gradually enhanced as we increased the concentrations of TGF- β 1, indicating a negative correlation between miR-378a expression and promoter methylation. The relationship between miR-378a expression and promoter methylation was also observed in primary HSCs isolated from CCl₄ rats and healthy controls. Interestingly, this negative correlation was additionally confirmed in patients with cirrhosis and healthy controls. Therefore, miR-378a expression is associated with promoter methylation in liver fibrosis. Notably, DNMTs including DNMT1, DNMT3a and DNMT3b were involved in the effects of TGF- β 1 on miR-378a methylation. In line with it, 5-Aza treatment caused a reduction in miR-378a methylation and induced an increase in miR-378a expression in TGF- β 1-treated LX-2 cells and TGF- β 1-treated primary HSCs. In sum, our results suggest that TGF- β 1-mediated DNMTs are involved in miR-378a methylation.

In conclusion, our results have provided new insights showing that miR-378a suppresses TGF- β 1-induced HSC proliferation and activation, at least in part, via targeting TGF- β 2. In addition, down-regulation of miR-378a expression is associated with promoter methylation in TGF- β 1-treated HSCs.

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Disclosure Statement

None.

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