Effect of SMYD3 on the microRNA expression profile of MCF-7 breast cancer cells

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Abstract. SET and MYND domain containing 3 (SMYD3) is a histone methyltransferase (HMT) and transcription factor, which serves important roles in carcinogenesis. Numerous downstream target genes of SMYD3 have been identified in previous studies. However, the downstream microRNA (miRNA) s regulated by SMYD3 are yet to be elucidated. In the present study, the results of miRNA microarray demonstrated that 30 miRNA expression profiles were upregulated, whilst 24 miRNAs were downregulated by >2.0-fold in the SMYD3-overexpressed MCF-7 breast cancer cells. The HMT activity was demonstrated to be essential for SMYD3-mediated transactivation of miR-200c-3p and the overexpression of miR-200c-3p inhibited the transactivation effects of SMYD3 on myocardin-related transcription factor-A-dependent migration-associated genes. To our best knowledge, the current study is the first to report on the transcriptional regulation of SMYD3 on miRNAs, and miR-200c may be a downstream negative regulator of the SMYD3-mediated pathway in the migration of breast cancer cells. These results may provide a novel theoretical basis to understand the mechanisms underlying the initiation, progression, diagnosis, prevention and therapy of breast cancer.

Introduction

SET and MYND domain containing 3 (SMYD3) is a novel histone methyltransferase gene identified in hepatoma and colon carcinoma cells by Hamamoto *et al* (1). SMYD3 is located on human chromosome 1 and encodes two protein isoforms that are composed of 428 and 369 amino acids. Previous studies have demonstrated that SMYD3 is frequently overexpressed in numerous types of cancer cells, including hepatic, colon, gastric and cervical carcinoma, and breast cancer (2-4), whilst the expression levels were lower in the corresponding normal tissue. A number of previous studies have demonstrated that SMYD3 has vital roles in the process of tumor development via its functions as a histone methylation enzyme and a transcription factor (5,6). SMYD3 modifies chromatin structure by catalyzing the methylation of histone H3 at lysine 4 (H3K4), H4K20 and H4K5 (5,6). Also, SMYD3 regulates the transcription of target genes via associating with RNA polymerase II or HELZ RNA helicase and binding at the motif CCCTCC or GGAGGG in the promoter (1).

MicroRNAs (miRNAs) are small, non-coding, endogenous RNA molecules of 18-22 nucleotides that were first identified in *Caenorhabditis elegans*. miRNAs suppress gene expression by binding the targeted mRNA transcripts, which causes translational repression or mRNA degradation. Previous studies demonstrated that miRNAs serve important roles in tumorigenesis through the regulation of genes involved in cancer development and maintenance (7,8).

A number of studies have identified that histone methylation and miRNAs are essential in the initiation and progression of cancer (7-9). However, the association between SMYD3 and miRNAs is yet to be elucidated. To investigate this further, the current study analyzed the global regulatory effects of SMYD3 on miRNAs in breast cancer cells using miRNA microarrays and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Materials and methods

Cell lines and plasmids. The MCF-7 human breast cancer cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The plasmid pcDNA5-TO/TAP-DEST-SMYD3 was a gift from Professor Philip Tucker from the Institute for Cellular and Molecular Biology, University of Texas (Austin, TX, USA). The CON049 (GeneChem, Co., Ltd., Shanghai, China) plasmid was used as

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a negative control for short hairpin (sh)SMYD3 (GeneChem, Co., Ltd.). The SMYD3-ΔNHSC plasmid was constructed by deleting the 205-208th amino acids (Asn-His-Ser-Cys) of pcDNA5-TO/TAP-DEST-SMYD3. The miR-200c-3p mimic and its negative control were obtained from Qiagen, Inc., (Valencia, CA, USA).

Cell culture and transfection. The MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM/F-12; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Tianjin Kangyuan Biotechnology Co., Ltd., Beijing, China) at 37˚C in an atmosphere containing 5% CO_2. For plasmid transfection experiments, MCF-7 cells were cultured in DMEM/F-12 medium without added hormones at 60% confluence for 12 h, and then transfected using TurboFect™ *in vitro* transfection reagent (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's protocol. Following incubation for 6 h, the medium was removed and replaced with normal culture medium (DMEM/F-12 medium without hormones) for 24 h. Then, the RT-qPCR was performed as described below. For miR-200c-3p mimic transfection experiments, Lipofectamine® 2000 reagents (Invitrogen; Thermo Fisher Scientific, Inc.) were used following the manufacturer's protocol.

RNA extraction and complementary DNA (cDNA) synthesis. Briefly, total cellular RNA was extracted from cultured cells using TRIzol reagent according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.) and 2 μg total RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocols. miRNA was isolated using the miRcute miRNA isolation kit (#DP501; Tiangen Biotech, Co., Ltd., Beijing, China), followed by cDNA synthesis using SuperScript First-Strand Synthesis system (Invitrogen; Thermo Fisher Scientific, Inc.), but with the specific stem loop primer primers. The miRNA RT-PCR primer sequences were as follows: JH6-miR200c-3p, 5'-CTCAACTGGTGTCGTGGAG TCGGCAATTCAGTTGAGTCCATC-3'; JH7-miR200c-3p, 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGA GTCCATCA-3'; JH7-miR149-3p, 5'-CTCAACTGGT-GTCGTG GAGTCGGCAATTCAGTTGAGGGGAGTG-3'. The total RNA and dNTPs were incubated at 65˚C for 5 min, cooled on ice briefly. Subsequently, the miRNA specific primer, RNase inhibitor and reverse transcriptase were added. After being incubated at 25˚C for 10 min, 37˚C for 50 min and 70˚C for 15 min, the cDNA was synthesized and used as a template of qPCR.

miRNA microarray chip analysis. miRNA expression profiling microarray was completed using Agilent human miRNA (8x60K) V18.0 miRNA array (LC Sciences, LLC., Houston, TX, USA). The microarray probe sequence was derived from Sanger MiRBase version 15.0 (http://microrna.sanger.org). Each chip contained multiple quality control probes and employed dual-color chip to examine miRNA expression profiling in MCF-7 cells with overexpressed or normal endogenous expression of SMYD3. Probes were synthesized *in situ* with photosensitive photogenerated reagents. The sequence consisted of two fragments: A chemically modified oligonucleotide encoding fragment complementary to target miRNA; and an extension arm at the distance specific to the connected encoding sequence that reduced the hybridization spatial impairment. Slides were scanned on an Agilent microarray scanner (model G2565A; Agilent Technologies, Inc., Santa Clara, CA, USA) at 100 and 5% sensitivity settings. Agilent Feature Extraction software version 8.1 (Agilent Technologies, Inc.) was used for image analysis (10-12).

RT-qPCR of mRNA and miRNA. RT-qPCR was performed using a StepOne™ Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Bestar® SYBR-Green qPCR Mastermix was obtained from DBI Bioscience (http://www.xinghanbio.com/cpzs; Shanghai, China). The thermal profiles were 95˚C for 10 sec and 60˚C for 1 min. Melting curve analysis was performed for each PCR to confirm the specificity of amplification. At the end of each phase, fluorescence was measured and quantified. Data is presented as the relative expression level of mRNA following normalization to GAPDH or the relative expression levels of miRNA following normalization to U6 following calculations using the $2^{-\Delta\Delta Cq}$ method (13). The PCR primer sequences were as follows: GAPDH forward, 5'-ATTCAACGGCACAGTCA AGG-3' and reverse, 5'-GCAGAAGGGGCGGAGATGA-3'; zinc finger E-box binding homeobox (ZEB)1 forward, 5'-AAG GGCAAGAAATCCTGGGG-3' and reverse, 5'-CTCTGGTC CTCTTCAGGTGC-3'; ZEB2 forward, 5'-AAATGCACAGA GTGTGGCAAGG-3' and reverse, 5'-CTGCTGATGTGCGA ACTGTAGGA-3'; SMYD3 forward, 5'-AAGTTCGAACCGC CAAGAG-3' and reverse, 5'-AAGGCAGCGGTCGCAGA CGA-3'; myosin light chain 9 (MYL9) forward, 5'-GAGCCCA AGCGCCTTCT-3' and reverse, 5'-GTCAATGAAGCCATCA CGGT-3'; cysteine rich angiogenic induced 61 (CYR61) forward, 5'-AAGGGGCTGGAATGCAACTT-3' and reverse, 5'-TTGGGGACACAGAGGAATGC-3'; U6 forward, 5'-CTC GCTTCGGCAGCACA-3'; and reverse, 5'-AACGCTTCACG AATTTGCGT-3'; miR200c-3p forward, 5'-ACACTCCAGCT GGGTAATACTGCCGGGTAAT-3'; miR149-3p forward, 5'-ACACTCCAGCTGGGTCTGGCTCCGTGTCTTG-3'; and general reverse, 5'-TGGTGTCGTGGAGTCG-3'. All experiments were repeated three times.

miRNA target gene prediction. In total 5 online prediction software programs, including miRDB (http://mirdb.org/), TargetScan (http://www.targetscan.org/), DIANA-microT (http://diana.cslab.ece.ntua.gr/microT/), microRNA.org (http://www.microrna.org/microrna/home.do) and RNA22 (https://cm.jefferson.edu/rna22), were used to predict the target genes of miRNAs. The intersection of 3/5 of these software programs were selected as the potential target genes.

Statistical analysis. Statistical evaluations were performed using GraphPad Prism (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA), using 3 independent experiments and were analyzed using Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference. The homologous alignment analysis was performed using DNAMAN (version 6.0; Lynnon LLC., San Ramon, CA, USA) and Primer Premier (version 5.0; Premier Biosoft International, Palo Alto, CA, USA).

Results

Effects of SMYD3 overexpression on the miRNA expression profile of MCF-7 cells. To investigate the regulatory effects of SMYD3 on global miRNA in breast cancer cells, MCF-7 cells were transfected with SMYD3 and microarray analysis was performed using the Agilent human miRNA (8x60K) V18.0 microarrays chip that contains 1,871 miRNAs. As presented in Fig. 1A, the expression profile analysis identified 1,871 microRNAs with altered expression, including 191 miRNAs that were upregulated in SMYD3-transfected cells compared with mock transfected cells, whilst 1,680 miRNAs were downregulated. Amongst these, 30 miRNAs were upregulated >2.0-fold and 24 miRNAs were downregulated <2.0-fold (Fig. 1B and C). Furthermore, to investigate the function of these miRNAs in more depth, the potential target genes of these miRNAs and their functions were summarized using bioinformatic analysis (Table I) (14-57).

Verifying the transcriptional regulatory effect of SMYD3 on the target miRNAs, miR-200c-3p and miR149-3p. To confirm the results of miRNA microarray, miR-200c-3p, a predicted target that was upregulated by 37.9-fold, and miR149-3p, a predicted target that was downregulated by 20.9-fold by SMYD3 in the chip assay, were selected for further investigation. The pcDNA5-TO/TAP-DEST-SMYD3 plasmid and the shSMYD3 plasmid were transfected into MCF-7 cells for the overexpression and knockdown of SMYD3, respectively. In accordance with the results of miRNA microarray, the results of RT-qPCR identified that miR-200c-3p was significantly upregulated following SMYD3 overexpression ($P<0.001$) and downregulated following RNA interference (RNAi) -mediated suppression of SMYD3 ($P<0.05$; Fig. 2A and B), whereas the level of miR149-3p exhibited a significant negative association with the expression of SMYD3 ($P<0.001$; Fig. 2C and D).

The promoter sequence of miR-200c-3p from a number of species, including of *Homo sapiens*, *Macaca mulatta*, *Pan troglodytes*, *Monodelphis domestica*, *Equus caballus* and *Rattus norvegicus*, were analyzed using bioinformatic methods. The results identified that 8 conserved SMYD3 binding sites exist in the promoter region (-2500 to +1) of miR-200c-3p from numerous species, indicating that miR-200c-3p may be a target for the transcriptional regulation of SMYD3 (Fig. 3).

Furthermore, the results of RT-qPCR assay demonstrated that the mRNA level of ZEB1 and ZEB2, two predicted target genes of has-miR-200c-3p presented in Table I, was decreased by SMYD3-overexpression, whereas they were increased by RNAi-induced suppression of endogenous SMYD3 (Fig. 4A and B). Taken together, these results demonstrate that SMYD3 activates the transcription of miR-200c-3p and therefore indirectly decreases the mRNA levels of ZEB1/2.

Histone methylation activity is essential in the SMYD3-mediated transactivation of miR-200c-3p. Considering the results of the present study and the previous studies that focusing on the histone methylation activity of SMYD3, it was hypothesized that SMYD3 may function as a transactivator of miR-200c-3p via its histone methylation function. To investigate this, MCF-7 cells were treated with 100 μM methylthioadenosine, a histone methylation inhibitor, with

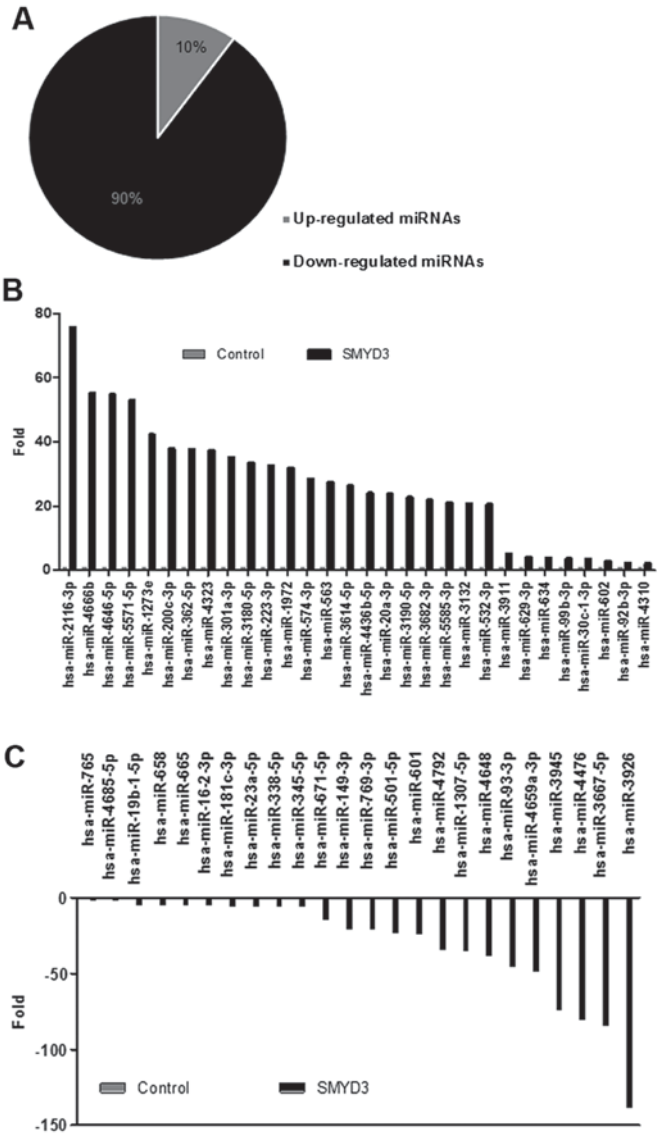

Figure 1. Expression profile of miRNAs following the overexpression of SMYD3 in MCF-7 cells. (A) The percentage of differentially expressed miRNAs following the overexpression of SMYD3 in MCF-7 cells. (B) The 30 miRNAs that were upregulated by >2.0-fold. (C) The 24 miRNAs that were downregulated by >2.0-fold. miRNA/miR, microRNA; SMYD3, SET and MYND domain containing 3.

or without physiological concentration of estradiol (10^{-11} M) and subsequently the transcription level of miR-200c-3p was detected using RT-qPCR. As presented in Fig. 5, the transcription level of miR-200c-3p was significantly downregulated by the histone methylation inhibitor (Fig. 5A). Consistently, SMYD3-ΔNHSC, a histone methyltransferase-activity-depleted mutant of SMYD3 (58,59), also decreases the transcription level of miR-200c-3p (Fig. 5B). These results suggested that the histone methylation activity may have vital roles in SMYD3-mediated transactivation of miR-200c-3p.

Regulatory effects of SMYD3 and miR-200c-3p on target genes of the RhoA-myocardin-related transcription factor A (MRTF-A) signaling pathway. The authors' previous study demonstrated that SMYD3 promotes MRTF-A-mediated transactivation of MYL9 and migration of MCF-7 breast

Table I. Functional classification of miRNAs and their potential target genes altered by SMYD3 in MCF-7 cells.

miRNA	Fold change	Functions	Predicted target genes
Upregulated miRNAs			
1 hsa-miR-2116-3p	75.68	Carcinogenesis of HCC	PEA15; ZDHHC11; TNRC6B; CFLAR; MTCH2
2 hsa-miR-4666b	55.23	Prostate cancer metastasis	VEZF1; EP300; SLC5A12; USP46
3 hsa-miR-4646-5p	54.83	Oncogenic stress, heat shock response and TLR pathway	CLIP3; SLC5A3; TGFBR1
4 hsa-miR-5571-5p	52.98	Sjögren's syndrome	PGM2L1; ATRN; RAD23B; ZNF10; NFAT5
5 hsa-miR-1273e	42.31	Fas apoptotic inhibitory, cell cycle, lysosomal/endosomal systems	ZC3H6; GAB1; GABPA; PDK3; AAK1; KLF12
6 hsa-miR-200c-3p	37.92	Migration, invasion, EMT and radiosensitivity in cancer cells	ZEB2; ZEB1; VASH2; RECK; ERRFI1; CCNJ; LHFP; LOX; CDK17; CYP1B1; SESN1
7 hsa-miR-362-5p	37.80	Cell proliferation and apoptosis resistance in gastric cancer and acral melanoma	LUC7L3; ACE2; STRN3; CHSY1; SYS1
8 hsa-miR-4323	37.41	Dedifferentiation	CAMK2G; HDGF; AFF4; ATXN7L3; PPP1R9B
9 hsa-miR-301a-3p	35.23	Placenta growth factor, NF-κB activator in pancreatic cancer cells and a T cells activator in EAE	TSC1; DDX6; MIER1; EIF2C4; FMR1; SLAIN1; MYBL1; ZFPM2; ENPP5
10 hsa-miR-3180-5p	33.62	Triple negative breast cancer, ulcerative colitis	CDR1; KCNJ3; AKIRIN1; ACTR2; SAMD5
11 hsa-miR-223-3p	32.69	Gastric cancer, recurrent ovarian cancer, eosinophil maturation, IGF-1R signaling and tumor suppression	FBXW7; SP3; FBXO8; CRIM1; IL6ST; LMO2
12 hsa-miR-1972	31.95	Lung cancer's early diagnosis, enterovirus 71 (EV71) infection	SLC41A1; DOPEY1; CYP27B1
13 hsa-miR-574-3p	28.42	Chondrogenesis	EP300; CUL2; SAMD4A; MESDC1
14 hsa-miR-563	27.50	Oxidative stress response	COL1A2; RNF111; SNX24; COL3A1
15 hsa-miR-3614-5p	26.42	Breast cancer susceptibility	CPEB3; BBX; PTPRD; NF2
16 hsa-miR-4436b-5p	24.11	Prion diseases and transcriptional misregulation in cancer	RBM26; FAM53C; NAV3
17 hsa-miR-20a-3p	23.86	Carcinogenesis of gastric cancer and ovarian cancer	SLC38A2; RNF19A; CADPS2; WDR47; CDH13
18 hsa-miR-3190-5p	22.82	Breast cancer and irritable bowel syndrome susceptibility	ARID3B; ZNF704; LIMD1; ZC3H7B
19 hsa-miR-3682-3p	21.96	TLR pathway; gastrointestinal cancer	ARMCX3; FXR1; MKL2; SHISA9; EPC2; TRDMT1
20 hsa-miR-5585-3p	21.16	Cellular cycle, apoptosis, stress responses	NRG3; IGF1; ARHGAP5; XRCC4; MEX3B; RBM24
21 hsa-miR-3132	20.75	Differential immune responses following hantavirus infection	ICAM5; TMEM132E; MTUS2; BLID
22 hsa-miR-532-3p	20.65	Apoptosis process	CYTH1; OTUD7B; GSG1L; HECTD1; DTX4
23 hsa-miR-3911	5.13	Oral carcinogenesis	SSR1; BCOR; RAB6B; SNTB2
24 hsa-miR-629-3p	4.13	Systemic lupus erythematosus and lung cancer	TP53INP2; ADIPOR2; MYOCD; ZNF322
25 hsa-miR-634	3.87	Regulating glioblastoma cell proliferation	PDIK1L; CERK; TBC1D8; CRISPLD2

Table I. Continued.

miRNA	Fold change	Functions	Predicted target genes
26 hsa-miR-99b-3p	3.73	AKT/mtor signaling pathway	TMEM87A; RANBP6; ZNF664
27 hsa-miR-30c-1-3p	3.62	Doxorubicin-resistant in human breast cancer cell	PCDHAC2; PCDHA6; PCDHA4; PCDHA11
28 hsa-miR-602	2.91	Acute rejection and an early stage of HBV-mediated hepatocarcinogenesis	NOG; HTT; ORMDL1; HABP4; CSK
29 hsa-miR-92b-3p	2.35	Hepatocarcinogenesis and G1/S phase of cell cycle	CD69; SLC12A5; FBXW7; MYO1B; DCAF6; RAB23; FNIP1; MAP2K4; RNF38; ACTC1
30 hsa-miR-4310	2.18	Nitrate transport	EPHA7; PLS3; ANO3; ANKIB1; RWDD4; CAB39; KITLG; NOVA1
Downregulated miRNAs			
1 hsa-miR-765	-2.24	Squamous cell carcinoma and traumatic brain injury and neural tube defects	PLP2; TIMP3; ARID1A; CHRDL1; MYO1A
2 hsa-miR-4685-5p	-2.31	NF-κB signaling pathway and TLR pathway	NHSL1; THPO; DIAPH1; CHAC1; USP42; PPP3CB
3 hsa-miR-19b-1-5p	-4.91	5-FU-resistant and tumorigenicity	EDNRB; RBMS3; PHF20
4 hsa-miR-658	-4.95	Acute rejection, lupus nephritis and gastric cancer	RASD2; CNTNAP1; DPYSL5; PPP1R16B
5 hsa-miR-665	-5.59	DNA hypermethylation and immune response; breast cancer, sporadic amyotrophic lateral sclerosis and mesial temporal lobe epilepsy	TGFBR1; GFI1B; ALKBH5; PFKFB2; COPS7B
6 hsa-miR-16-2-3p	-5.61	P53 activation, doxorubicin resistant, tumor cell proliferation and apoptosis, polycythemia vera	RAB6A; INTU; SGIP1; SP8
7 hsa-miR-181c-3p	-5.81	Wnt/b-catenin signaling, neuronal apoptosis, methylation	FAM122B; NHEJ1
8 hsa-miR-23a-5p	-5.87	Glutamine metabolism, EMT	PPA2; VSIG1; SF3B1; FOXC1
9 hsa-miR-338-5p	-5.93	HCC, androgen	PHC3; STAG2; SP3; TNPO1; RAB28; BMI1
10 hsa-miR-345-5p	-6.14	Multidrug resistance, cell proliferation and invasion	SUV420H1; CTTNBP2NL; RPA1; RFC1
11 hsa-miR-671-5p	-14.66	Non-coding antisense transcripts and gene silencing	LIN9; SLC30A6; TBL2
12 hsa-miR-149-3p	-20.94	EMT, proliferation, metastasis and cell cycle	SMARCD; YBX2; MS4A4A; UBE2Q1; SRRM2; CNGA2
13 hsa-miR-769-3p	-21.45	Adenosine deamination; Nervous system- related and tumor-related biological processes and signaling pathways	PSMD14; GPC4; TMEM139; GAP43
14 hsa-miR-501-5p	-23.13	Apoptosis, tumorigenesis	LPAR1; DNAJC6; KIAA202; RNF165; PEX12; JMY
15 hsa-miR-601	-24.18	The negative regulation of translational initiation, ESCC and CRC	LHFPL2; SNN; ZBTB38; SIRT1
16 hsa-miR-4792	-34.45	HIV infection and infectious pancreatic necrosis virus	PCDH19; RNF214; MAPK9; FRMPD4

Table I. Continued.

miRNA	Fold change	Functions	Predicted target genes
17 hsa-miR-1307-5p	-35.72	Metastasis	COL22A1; DPP4
18 hsa-miR-4648	-38.77	Brain specific	YWHAZ; SLC9A2; BOLL; TNPO3
19 hsa-miR-93-3p	-46.09	Tumor proliferation and differentiation angiogenesis and metastasis	SYNJ1; AEBP2; TARDBP; GLCCI1
20 hsa-miR-4659a-3p	-49.33	Immune response	ASCL1; CSNK1G1; ZBTB39; KCND2; PRRX1
21 hsa-miR-3945	-74.33	Traumatic pathogenesis, cerebrovascular disease	FNDC3B; RIC8B; KBTBD2; BIRC6
22 hsa-miR-4476	-80.76	Myocardial damage	POU2AF1; ATP6AP1; C9ORF152; DPYSL2; LENG8; RANBP10
23 hsa-miR-3667-5p	-84.87	Inflammation	RAP1A; CPEB3; CMTM6; MYCBP; HOXD10
24 hsa-miR-3926	-139.10	TLR pathway and prominin-1/CD133	KCNG3; PIGA; RBMXL1

Fold ≥0 indicated that the expression level of the miRNA was upregulated, whilst the fold ≤0 indicated that the expression level of the miRNA was downregulated. miR/miRNA, microRNA; SMYD3, SET and MYND domain containing 3.

cancer cells (59). However, other previous studies have identified that miR-200c represses migration and invasion of breast cancer cells via suppression of the RhoA-MRTF-A signaling pathway (60,61). As the results of the present study demonstrated the overexpression of SMYD3 directly transactivates miR-200c-3p, whether SMYD3 and miR-200c-3p supports or opposes each others' effects on the transcription of MYL9 and CYR61, two MRTF-A-dependent migration-related genes, was investigated. The miR-200c-3p mimic and/or SMYD3-overexpression plasmid were transfected into MCF7 cells and the transcription level of MYL9 and CYR61 were detected using RT-qPCR. The results of RT-qPCR demonstrated that the transcriptional level of SMYD3 and the miR-200c-3p increased significantly following the transfection (Fig. 6A and B). As presented in Fig. 6C, the mRNA levels of MYL9 and CYR61 were suppressed by miR-200c-3p and increased by the overexpression of SMYD3. Furthermore, the upregulation of MYL9 and CYR61 remained after SMYD3 was co-transfected with miR-200c-3p mimics, but the effect in combination group was reduced compared with that of the SMYD3-transfected group. These results demonstrated that miR-200c-3p may be a downstream negative regulator of the SMYD3-mediated pathway in the migration of breast cancer cells.

Discussion

The primary focus of epigenetics is to elucidate the heritable changes in gene expression and regulation, which occur without mutation to the DNA sequence, in the process of gene transcription in eukaryotic cells. The heritable alterations include DNA methylation, histone modification and regulation by noncoding RNAs. A number of studies have established that the majority of malignant tumor types exhibit cancer-associated epigenetics alterations (62). However, the interdisciplinary investigation of miRNA and histone modification has received increasing interest.

A previous study has analyzed downstream coding genes of SMYD3 using cDNA microarray and the results identified that there were 61 genes upregulated >3-fold and genes downregulated <3-fold by SMYD3 (1). However, to date, the downstream miRNAs regulated by SMYD3 are yet to be elucidated. Therefore, the present study focused on the effect of SMYD3 on the miRNA expression profile in MCF-7 breast cancer cells by analyzing microarray and RT-qPCR data. These results demonstrated that 30 miRNAs were upregulated >2.0-fold, whilst 24 miRNAs were downregulated <2.0-fold following the overexpression of SMYD3. The analysis of bioinformatic and previously published data identified that the majority of these potential downstream miRNAs of SMYD3 were associated with the proliferation, migration and therapy resistance of tumor.

To further validate the results of microarray, RT-qPCR was performed to detect the effects of SMYD3 on the expression of miR-200c-3p and miR-149, which are 2 representative target miRNAs of SMYD3 with opposing alterations, and the results were in accordance with those of the microarray. Previous studies have demonstrated that miR-149 and miR-200c are associated with the progression of cancer (63-65). miR-149 has been established as a tumor suppressor by inhibiting the spreading, migration and invasion of basal-like breast cancer

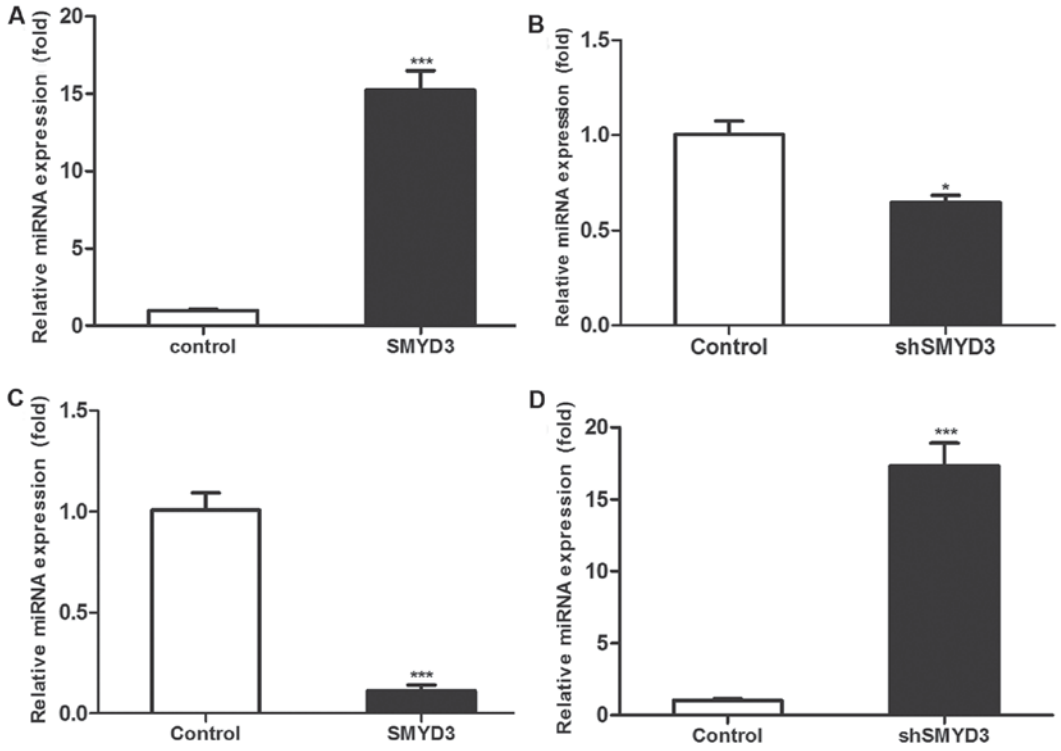

Figure 2. SMYD3 upregulates miR-200c-3p and downregulates miR149-3p in MCF-7 cells. The transcriptional levels of (A and B) miR-200c-3p and (C and D) miR149-3p in MCF-7 cells transfected with pcDNA5-TO/TAP-DEST-SMYD3 and shSMYD3 was detected using reverse transcription-quantitative polymerase chain reaction. *P<0.05, ***P<0.001. miRNA/miR, microRNA; SMYD3, SET and MYND domain containing 3; sh, short hairpin.

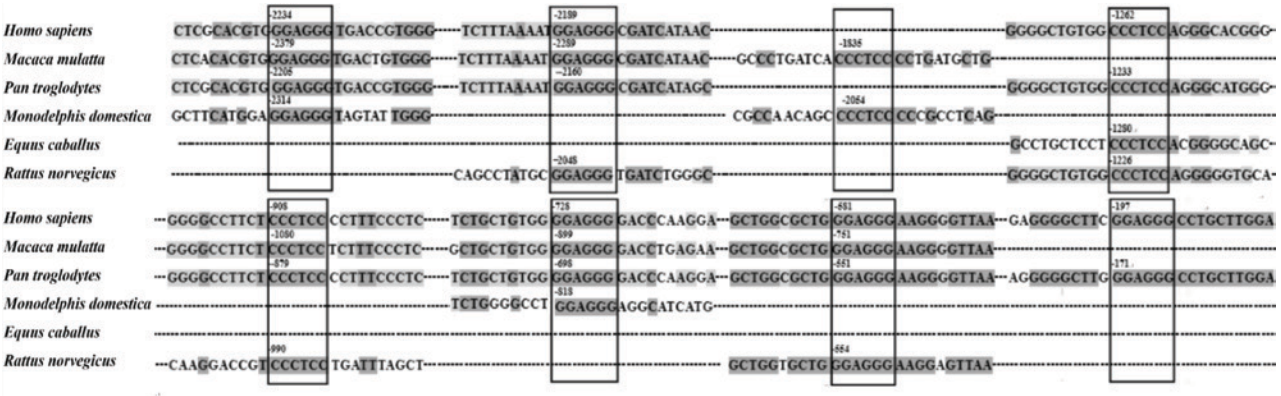

Figure 3. Conserved SMYD3 binding sites in miR-200c-3p promoter from numerous species. The homologous alignment analysis of the binding sides of SMYD3 in miR-200c-3p promoter from a number of species was performed using DNAMAN (version 6.0) and Primer Premier (version 5.0). miR, microRNA; SMYD3, SET and MYND domain containing 3.

cells (63). However, the roles of miR-200c in cancer remain to be fully understood. A number of previous studies have reported that miR-200c may inhibit the epithelial-mesenchymal transition and enhance the chemosensitivity and radiosensitivity of cancer cells (64,65), whereas another previous study reported a metastasis-promoting role of miR-200 in breast cancer (64). In addition, a separate study demonstrated that the expression level of miR-200c different between the subtypes of breast cancer (65). Compared with the normal breast epithelial cells (MCF-10A), the basal cancer cells (MDA-MB-231 and BT549) exhibited a reduced expression of miR-200c (65). However, the expression of miR-200c in luminal cancer cells (MCF-7 and BT474) was increased compared with that in the normal breast epithelial cells (65). Additionally, previous studies have suggested that miR-200c may repress migration and invasion of breast cancer cells via the suppression of RhoA-MRTF-A signaling pathways (60,61), whereas the current study suggests that SMYD3 promotes MRTF-A-mediated transactivation of MYL9 and migration of MCF-7 breast cancer cells (59). The results in the present study have demonstrated that the expression of miR-200c in MCF-7 breast cancer cells may be upregulated by SMYD3, and the overexpression of miR-200c partially inhibits the transactivation effects of SMYD3 on the MRTF-A-dependent migration-associated genes. Taken together, these results indicated that miR-200c may be a downstream negative regulator of the SMYD3-mediated pathway in the migration of breast cancer cells, and may promote a negative feedback loop to prevent excessive induction of migration

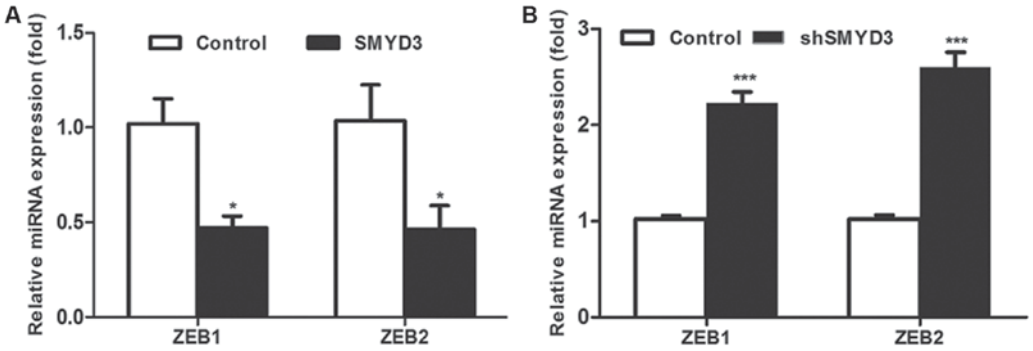

Figure 4. SMYD3 decreases the mRNA level of ZEB1/2, 2 predicted target genes of miR-200c-3p. (A) The mRNA level of ZEB1/2 in MCF-7 cells transfected with pcDNA5-TO/TAP-DEST-SMYD3 plasmid was detected using RT-qPCR. (B) The mRNA level of ZEB1/2 in MCF-7 cells transfected with shSMYD3 plasmid was detected using RT-qPCR. *P<0.05, ***P<0.001. miR/miRNA, microRNA; SMYD3, SET and MYND domain containing 3; ZEB, zinc finger E-box binding homeobox; sh, short hairpin; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

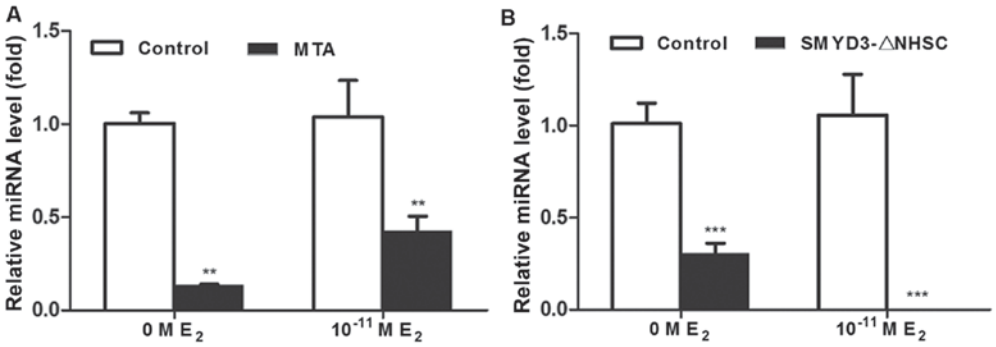

Figure 5. Histone methylation activity of SMYD3 has vital roles in promoting miR-200c-3p. (A) The difference of miR-200c-3p levels in MCF-7 cells treated by MTA with or without E_2 (10^{-11} M) was detected by RT-qPCR. (B) The difference of miR-200c-3p levels in MCF-7 cells transfected with SMYD3-ΔNHSC (Asn-His-Ser-Cys) plasmid and treated with or without E_2 (10^{-11} M) was detected by RT-qPCR. **P<0.01, ***P<0.001. miR/miRNA, microRNA; SMYD3, SET and MYND domain containing 3; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; MTA, methylthioadenosine; E_2, estradiol.

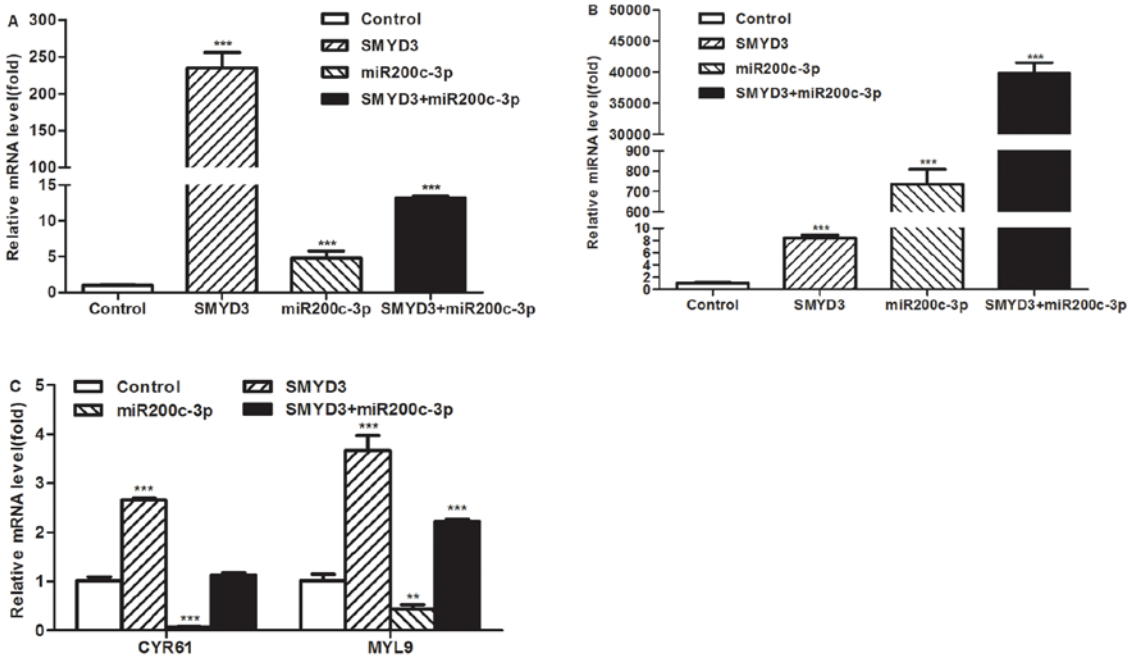

Figure 6. miR-200c-3p may be a downstream negative regulator of SMYD3-mediated pathway. (A) The transfection efficiency of SMYD3 was detected using RT-qPCR. (B) The transfection efficiency of miR-200c-3p mimics was detected using RT-qPCR. (C) miR-200c-3p mimics and/or SMYD3-overespression plasmids were transfected into MCF7 cells and the transcription levels of MYL9 and CYR61 were detected using RT-qPCR. **P<0.01, ***P<0.001. miR/miRNA, microRNA; SMYD3, SET and MYND domain containing 3; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CYR61, cysteine rich angiogenic induced 61; MYL9, myosin light chain.

of breast cancer cells. However, the underlying mechanism of miR-200c in breast cancer remains to be elucidated by future investigation.

Additionally, previous studies have identified that SMYD3 alters chromatin structure by catalyzing the di-/tri-methylation of histone H3 at lysine 4 (H3K4me2/3), H4K20me3 and H4K5me (5,6). As part of the established methylation marks at H3K4 or H3K36, mono-methylations of H3K9, H3K27, H3K79, H4K20 and H2BK5 are associated with transcriptional activation, whereas trimethylations of H3K9, H3K27, H3K79 and H4K20 are associated with transcriptional repression (5,6). The current study demonstrates that the histone methylation activity is essential for SMYD3-mediated transactivation of miR-200c-3p. Therefore, SMYD3 antagonistic regulation of the downstream miRNAs may also depend on the distinct modification on the histone adjacent to the promoter.

In conclusion, the effect of SMYD3 on the miRNA expression profile in MCF-7 breast cancer cells was analyzed using microarray and RT-qPCR in the present study. To the best of our knowledge, this is the first study focused on the transcriptional regulation of SMYD3 on miRNAs. These results may provide a novel theoretical basis to elucidate the mechanism underlying the initiation, progression, diagnose, prevention and therapy of breast cancer.

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