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Suppression of Wnt signaling by the miR-29 family is mediated by demethylation of WIF-1 in non-small-cell lung cancer

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ABSTRACT

Wnt inhibitory factor-1 (WIF-1) silencing induced by promoter hypermethylation is a common mechanism of aberrant activation of the Wnt signaling pathway in non-small-cell lung cancer (NSCLC). However, the activity of regulators associated with the methylation of the WIF-1 gene remains unclear. Here, we investigated the role of three DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) in the expression of WIF-1. The three DNMTs were up-regulated in NSCLC tumor tissues and suppression of DNMT3A and DNMT3B restored the expression of WIF-1 in NSCLC cells. The miR-29 family (miR-29a, -29b, and -29c), which negatively regulates DNMT3A and DNMT3B, was examined in association with the Wnt/β-catenin signaling pathway. A positive correlation between the expression of WIF-1 and that of MiR-29s was observed in NSCLC tissues. Methylation-specific PCR and Western blotting indicated that miR-29s positively regulate WIF-1 expression by inhibiting the methylation of its promoter. Furthermore, miR-29 overexpression downregulated β-catenin expression, inhibited cell proliferation and induced apoptosis. The expression of miR-29a and miR-29b was partially regulated by DNMT3A and DNMT3B in a positive feedback loop. Taken together, our findings show that miR-29s suppress the Wnt signaling pathway through demethylation of WIF-1 in NSCLC.

1. Introduction

The incidence rate of lung cancer has increased over the past decades in China in association with high mortality rates [1]. Non-small-cell lung cancer (NSCLC) is the major histological class accounting for 80% of all lung cancers. Despite advances in multimodality therapies, surgery remains the primary treatment for NSCLC patients with generally unsatisfactory results in advanced disease. New effective therapeutic strategies are urgently needed, and further research on the molecular mechanisms underlying lung cancer is essential.

Alterations in growth regulatory signaling pathways play an important role in the pathogenesis of NSCLC. Numerous reports have shown an association between aberrant activation of the Wingless-type (Wnt) signaling pathway and carcinogenesis including that of NSCLC [2,3]. The Wnt family is a group of secreted signaling molecules that mediate a variety of cellular processes through canonical and/or noncanonical pathways [4,5]. In the canonical pathway, the Wnt ligand binds directly to the cysteine-rich extracellular domain of Frizzled (Fz), resulting in β-catenin stabilization and accumulation in the cytoplasm. The cytoplasmic β-catenin translocates into the nucleus and binds to Tcf/Lef transcription factors, forming a heterodimeric complex that further activates the transcription of important downstream target genes. Aberrant Wnt/β-catenin signaling has been shown to promote cancer cell growth and is associated with poor prognosis in NSCLC [3,6]. Noncanonical pathways are referred to as β-catenin-independent Wnt signaling [5].

The down-regulation of Wnt antagonists is a common mechanism of aberrant activation of the Wnt signaling pathway. As a major antagonist, Wnt inhibitory factor-1 (WIF-1) is silenced by promoter methylation in NSCLC [7]. However, the mechanism underlying the over-methylation of the WIF-1 gene remains unclear. DNA methylation involves the transfer of a methyl group from the methyl donor *S*-adenosyl methionine to the 5′ position on the cytosine ring, and is accomplished by DNA methyltransferases (DNMTs). Three catalytically active DNMTs, DNMT1, DNMT3A and DNMT3B, have been identified in mammals [8]. The mRNA levels of DNMT1, DNMT3A, and DNMT3B are reportedly elevated in various malignancies [9–11] and have been correlated with hypermethylation of tumor suppressor genes [12].

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MicroRNAs (miRNAs), a class of small noncoding RNAs, play important roles in tumorigenesis by targeting mRNAs [13]. DNMT3A and -B are directly regulated by the miRNA (miR)-29 family (29a, 29b, and 29c) [14]. The expression of MiR-29 family members is down-regulated in lung cancer [15], which has been associated with promoter methylation and transcription factor regulation [16,17]. However, the association between DNMT up-regulation and over-methylation of the WIF-1 gene and whether restoration of miR-29s suppresses Wnt signaling by modulating the methylation of WIF-1 remains unclear. In addition, the potential self-regulation of the expression of miR-29 family members through DNMT3A and -B needs clarification.

In the present study, we investigated the roles of three DNMTs in the expression of WIF-1 and linked miR-29s to the Wnt/β-catenin signaling pathway, investigating their effects on cell growth and apoptosis. We showed that the expression of miR-29a and miR-29b is regulated by DNMT3A and DNMT3B, suggesting the existence of a positive feedback loop. Our findings shed light on the role of aberrant Wnt signaling in NSCLC.

2. Materials and methods

2.1. Patient and tissue samples

Paired NSCLC and adjacent non-tumor lung tissues were obtained from 30 patients who underwent primary surgical resection of NSCLC with informed consent between March 2010 and March 2011 at Shanghai Tenth People's Hospital (China). Tissue samples were immediately frozen in liquid nitrogen after resection and stored at −80 °C until use. Both tumor and non-tumor samples were confirmed by pathological examinations. This study was approved by the Human Research Ethics Committee of Shanghai Tenth People's Hospital. The clinical stage was defined according to the revised International Staging System [18].

2.2. Cell culture and treatment

The human lung cancer cell lines A549 and H1299 were obtained from Shanghai Cell Bank, Chinese Academy of Sciences. The cells were maintained in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (Gibco) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37 °C.

Transfections with has-miR-29a, -29b-1, -29c mimics and negative control (Ambion, Austin, TX, USA) were performed at a concentration of 100 nM with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The expressions of DNMT1, DNMT3A and DNMT3B were knocked down stably in A549 and H1299 cell lines using a lentiviral shRNA system from Santa Cruz Biotechnology (Dallas, Texas, USA) with puromycin selection. Transient knock down of WIF-1 was achieved by using WIF-1 shRNA Plasmid (Santa Cruz) according to the manufacturer's instructions. For the demethylation assay, 5-Aza-deoxycytidine (5-Aza, Sigma–Aldrich, St. Louis, MO, USA) was added at a concentration of 10 μM for 72 h.

2.3. Real-time PCR

Total RNA was extracted from tissues and cells using the Trizol reagent (Invitrogen). mRNA levels were analyzed by real time-PCR after reverse transcription with Superscript III reverse transcriptase (Invitrogen) using SYBR Premix Ex Taq™ (Takara, Otsu, Shiga, Japan) in the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. MicroRNA levels were quantified using the TaqMan MicroRNA assay kit (Applied Biosystems) according to the manufacturer's protocol. Relative expression of mRNA was normalized to the expression of β-actin mRNA and U6 small RNA and calculated with the $2^{-\triangle\triangle Ct}$ method. The primers for DNMT1, DNMT3A and DNMT3B, WIF-1 and β-actin are listed in Table 1. All experiments were performed in triplicate.

2.4. Western blotting

Frozen tissue samples were pulverized and placed in homogenization buffer (10 mM phosphate buffer, 250 mM sucrose, 1 mM EDTA, 0.1 mM PMSF, and 0.1% tergitol, pH 7.5). Homogenates were centrifuged at 27,000*g* for 10 min at 4 °C, and the supernatant was isolated as the total protein fraction. Cells were lysed in ice-cold lysis buffer containing 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and complete proteinase inhibitor mixture. The proteins were separated by SDS–PAGE, transferred to a PVDF membrane, and then incubated with the following primary antibodies: rabbit anti-WIF-1, mouse anti-DNMT1, rabbit anti-DNMT3A, mouse anti-DNMT3B (all from Abcam plc., Cambridge, MA, USA, 1:1000 dilution), and rabbit anti-PARP, anti-β-catenin and anti-β-actin antibodies (all from Cell Signaling Technology, Inc., Danvers, MA, USA, 1:1000 dilution). After incubation with HRP-conjugated secondary antibodies (Cell Signaling), protein bands were visualized using the Pierce ECL Western blotting substrate (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.5. Methylation-Specific PCR (MSP)

Genomic DNA was extracted with the DNA Maxi Kit (Qiagen, Valencia, CA), and modified with sodium bisulfite using the

Table 1
Primer sequences.

Gene	Sequence (5′–3′)	
	Forward	Reverse
Realtime-PCR primer		
WIF-1	CCTGGATTCTATGGAGTGAACTG	TCTAGTCCTGGAGGGCAAATA
DNMT1	CCAGGATGAGAAGAGACGTAGA	AGTGCGCGTTCCTGATTT
DNMT3A	GCCCATTCGATCTGGTGATT	GGCGGTAGAACTCAAAGAAGAG
DNMT3B	AAGAGTTGGGCATAAAGGTAGG	CCTCACGTCGTTCACGTATT
β-actin	CACTCTTCCAGCCTTCCTTC	GTACAGGTCTTTGCGGATGT
MSP primers		
Methylated		
WIF-1	GGGCGTTTTATTGGGCGTAT	AAACCAACAATCAACGAAC
miR-29b1-miR-29a	CGGTTTTTTAGTATTATTGTTAGTCGT	AAAACACATATCAACCCCGTC
miR-29b2-miR-29c	TATAGTTATATGTTGGGGAAAGACG	ATAAAACAAAAAATTCCTAAACGAT
Unmethylated		
WIF-1	GGGTGTTTTATTGGGTGTAT	AAACCAACAATCAACAAAAC
miR-29b1-miR-29a	TGGTTTTTTAGTATTATTGTTAGTTGT	AAAAACACATATCAACCCCATC
miR-29b2-miR-29c	TAGTTATATGTTGGGGAAAGATGG	ATAAAACAAAAAATTCCTAAACAAT

MethylCode Bisulfite Conversion Kit (Invitrogen) according to the manufacturer's protocol. The methylation-specific or unmethylation-specific primer (MSP or USP) for WIF-1 was designed according to a previous report [7] (Table 1). MiR-29s are transcribed into two primary transcripts from two chromosomes (miR-29b1and miR-29a at chromosome 7q32; miR-29b2 and miR-29c at chromosome 1q32). The MSP and USP were designed using the MethPrimer program (http://www.urogene.org/cgi-bin/methprimer/methprimer). Primers for miR-29b1 and miR-29a correspond to the promoter region sequences −1112 to −1085 and −993 to −971, respectively (Table 1). Primers for miR-29b2 and miR-29c correspond to the promoter region sequences −1339 to −1314 and −1178 to −1153, respectively (Table 1).

2.6. Cell proliferation assays

Cancer cells were transfected with microRNA mimics and/or WIF-1 shRNA. Seventy-two hours after transfection, cells were seeded into 96-well plates (5×10^3 cells per well), and incubated at 37 °C. The number of viable cells was measured at daily intervals. A volume of 10 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and incubated for 4 h. The precipitate formed was dissolved by addition of 100 μL of dimethyl sulfoxide and absorbance was measured at 562 nm using an ELISA reader (Bio-Rad Laboratories, Hercules, CA, USA). Each experiment was performed in triplicate and repeated three times.

2.7. Apoptosis assay

Cells were seeded in 6-well plates at a density of 1×10^6 cells per well and transfected with microRNA mimics and/or WIF-1 shRNA. Seventy-two hours after transfection, the cells were resuspended and stained with FITC-conjugated anti-annexin V antibody and propidium iodide (PI) using the Annexin V-FITC Apoptosis Detection Kit (Sigma–Aldrich). Stained cells were then quantified by FACSCalibur flow cytometry (Becton Dickinson, USA).

2.8. Statistical analysis

Differences between two groups were assessed by the Student's *t*-test or Mann–Whitney's *U*-test. Correlations were assessed by Pearson's correlation test. Variance analysis between multiple groups was performed by one-way ANOVA. The data were presented as mean ± standard deviation (SD) and $p < 0.05$ was considered statistically significant.

3. Results

3.1. DNMT3A and DNMT3B catalyze the methylation of WIF-1 in NSCLC

The expression level of WIF-1, DNMT1, DNMT3A and DNMT3B was detected in 30 paired NSCLC and adjacent non-neoplastic lung tissues by real-time PCR and Western blotting. As shown in Fig. 1A and E, WIF-1 was expressed at significantly lower levels in tumor tissues than in matched non-tumor tissues. In contrast, the expression of DNMTs (DNMT1, DNMT3A and DNMT3B) was up-regulated in cancerous lung tissues (Fig. 1B–D and F–H).

The role of the three DNMTs in the methylation of the WIF-1 gene was verified by lentiviral vector mediated knockdown of these methyltransferases in A549 and H1299 cells and assessment of WIF-1 expression. The results of Western blotting confirmed the downregulation of DNMT expression and showed a significant increase in WIF-1 protein levels in cells transfected with DNMT3A or DNMT3B shRNA (Fig. 1I and J). These results indicated that DNMT3A and DNMT3B play a role in the regulation of WIF-1 gene expression in NSCLC.

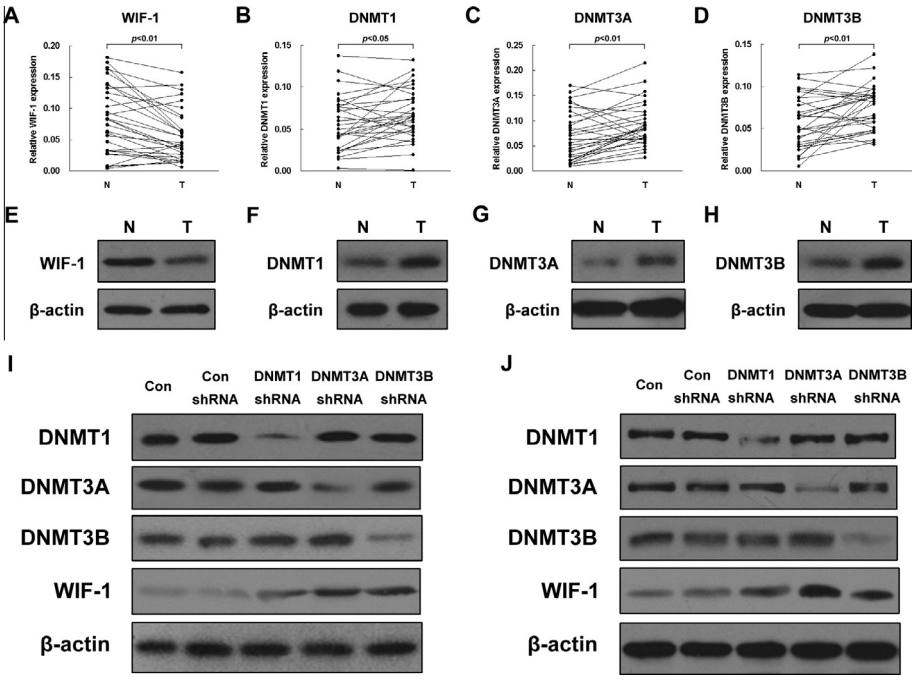

Fig. 1. DNMT3A and DNMT3B catalyze the methylation of WIF-1 in NSCLC. (A–D) Relative mRNA expression of WIF-1, DNMT1, DNMT3A and DNMT3B in NSCLC tissues ($n = 30$) and in paired non-tumor tissues ($n = 30$). (E–H) Representative gels showing the protein levels of WIF-1, DNMT1, DNMT3A and DNMT3B in 30 tumor tissue samples and paired non-tumor tissues. (I and J) shRNA-mediated knockdown of DNMT3A and DNMT3B restores the expression of WIF-1. A549 cells (I) or H1299 cells (J) were stably transfected with negative control shRNA (Con shRNA), DNMT1 shRNA, DNMT3A shRNA and DNMT3B shRNA, and the protein levels of DNMTs and WIF-1 were assessed by Western blotting.

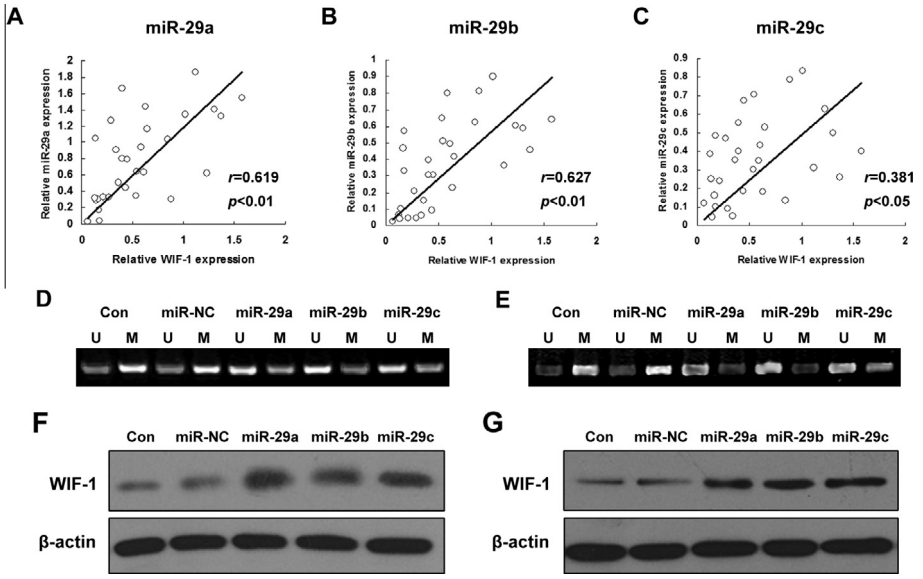

Fig. 2. Effect of restoration of miR-29s on the expression of WIF-1. (A–C) Correlation between endogenous miR-29 and WIF-1 mRNA levels in 30 NSCLC tissues. *r*, regression coefficient. (D and E) Restoration of the expression of miR-29s inhibits the methylation of the WIF-1 promoter in A549 (D) and H1299 (E) cells. Cells were transfected with miR-29a, -29b, -29c mimics and a negative control (miR-NC). The methylation status of the WIF-1 promoter was analyzed by methylation-Specific PCR. (F and G) Restoration of miR-29 expression upregulates the expression of WIF-1 in A549 (F) and H1299 (G) cells. WIF-1 protein levels were detected by Western blotting after transfection of A549 or H1299 cells with miR-29s or a negative control (miR-NC).

3.2. Restoration of miR-29s positively regulates WIF-1

To examine the mechanism of up-regulation if DNMTs, we focused on the effect of the miR-29 family because miR-29s are down-regulated in NSCLC and target DNMT3A and -3B. To determine whether WIF-1 mRNA expression is correlated with the levels of miR-29s in NSCLC tissues, the mRNA levels of WIF-1 and miR-29s were analyzed in 30 NSCLC samples. The results showed statistically significant positive correlations (Fig. 2A–C) between WIF-1 mRNA and miR-29a ($p < 0.01$), miR-29b ($p < 0.01$) and miR-29c ($p < 0.05$).

To confirm that expression of miR-29s contributes to the reduction of promoter methylation of the WIF-1 gene, we examined the methylation status of WIF-1 using MSP. As shown in Fig 2D and E, reduced methylation of WIF-1 was observed in A549 and H1299 cells transfected with miR-29a, -29b, or -29c. Furthermore, Western blot analysis confirmed that enforced expression of these miRNAs increased the protein level of WIF-1 in A549 and H1299 cells (Fig. 2F and G).

3.3. MiR-29s inhibit the Wnt/β-catenin signaling pathway by restoring the expression of WIF-1

The upregulation of WIF-1 expression by miR-29s suggested that these miRNAs suppress Wnt signaling in NSCLC. In a previous study, inhibition of Wnt signaling was shown to induce apoptosis and inhibit tumor growth in lung cancer cell lines [19,20]. We therefore examined the effect of overexpression of miR-29s on the protein levels of β-catenin, cell proliferation and apoptosis. As shown in Fig. 3A and B, overexpression of miR-29s decreased the levels of the β-catenin protein, indicating that Wnt signaling was repressed by miR-29s.

The MTT assay showed that cell proliferation was significantly inhibited in cells transfected with miR-29a, -29b, or -29c (Fig. 3C and D). Western blotting and flow cytometry analyses showed that overexpression of miR-29s promoted the cleavage of PARP (Fig. 3E and F) and increased the rate of apoptosis (Fig. 3G and H) in A549 and H1299 cells.

To confirm that the effect of miR-29s is mediated by the regulation of WIF-1 expression, shRNA knock-down of WIF-1 was performed in cells transfected with miR-29a, -29b, -29c mimics. The results showed that silencing of WIF-1 expression markedly offset the effect of miR-29s (Fig. 3).

3.4. Self-regulation of miR-29a and miR-29b expression is mediated by DNMT3A and DNMT3B

To further investigate whether the expression of miR-29s is regulated by methylation, NSCLC cells were exposed to 5-Aza, a demethylating agent, which partly restored miR-29a and miR-29b levels (Fig. 4A and B). Furthermore, the expression of miR-29a and miR-29b was upregulated in cells transfected with DNMT3A or DNMT3B shRNA (Fig. 4C and D). To confirm that the expression of miR-29a and miR-29b is regulated through demethylation mediated by DNMT3A and -3B, we examined the methylation status of miR-29a and miR-29b. As shown in Fig 4E and F, methylation of the miR-29b1-miR-29a promoter was decreased in A549 and H1299 cells transfected with DNMT3A or -3B shRNA. However, the miR-29b2-miR-29c promoter showed a low methylation status, implying that miR-29b2 and miR-29c may not be regulated by methylation.

4. Discussion

Concurrent aberrant activation of the Wnt pathway and downregulation of Wnt antagonists are common in cancer cells [21,22]. Silencing or downregulation of the WIF-1 gene by promoter hypermethylation has been detected in several malignancies including lung cancer, and it has been associated with tumorigenesis [7,23–26]. In the present study, three DNMTs (DNMT1, DNMT3A and DNMT3B) were found to be up-regulated in NSCLC tumor tissues and their suppression restored the expression of WIF-1 in NSCLC cells.

Because DNMT3A and DNMT3B are negatively regulated by miR-29s, we explored the involvement of the miR-29 family in the aberrant regulation of Wnt signaling. Mir-29 family members

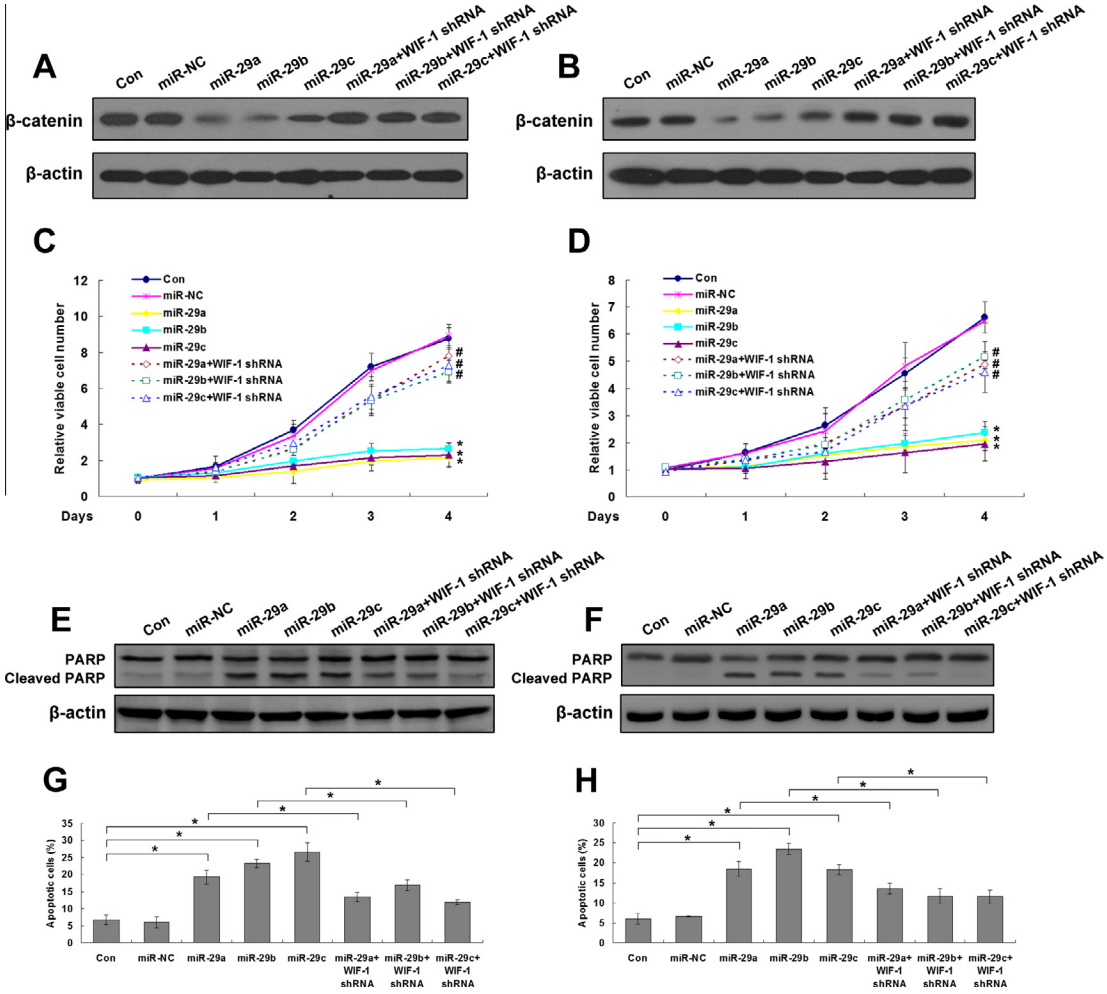

Fig. 3. MiR-29s suppress Wnt/β-catenin signaling by restoring the expression of WIF-1. A549 cells or H1299 cells were transfected with miR-29a, -29b, -29c mimics or co-transfected with miR-29 and WIF-1 shRNA. (A and B) Restoration of the expression of miR-29s decreased β-catenin protein levels by up-regulating WIF-1 in A549 (A) and H1299 (B) cells. β-catenin protein levels were detected by Western blotting. (C and D) Over-expression of miR-29 family proteins inhibits the proliferation of A549 (C) and H1299 (D) cells. Cells were transfected as indicated and cell proliferation was assessed using the MTT assay at the indicated time points. $^{*}p < 0.05$ compared with control (Con), $^{\#}p < 0.05$ compared with cells transfected with miR mimics. (E and F) Restoration of the expression of miR-29s increases PARP cleavage in A549 (E) and H1299 (F) cells. PARP cleavage was examined by Western blotting. (G and H) Restoration of the expression of miR-29s induces apoptosis in A549 (G) and H1299 (H) cells. Apoptosis was assessed by flow cytometric analysis. $^{*}p < 0.05$. Experiments were performed in triplicate and values are expressed as mean ± SD.

have been described as tumor suppressor genes and their expression is down-regulated in several cancers, including NSCLC [14], cholangiocarcinoma [27] and acute myeloid leukemia [28]. We observed that the expression of miR-29s was positively correlated with the expression of WIF-1 in NSCLC tissue. The results of MSP and Western blotting indicated that miR-29s contribute to the reduction of promoter methylation of the WIF-1 gene and positively regulate the expression of WIF-1. These results confirmed our hypothesis and link miR-29s to the Wnt pathway.

In the present study, we showed that miR-29s inhibited Wnt/β-catenin signaling as shown by the effect of overexpression of miR-29 family members on the downregulation of β-catenin expression. In agreement with previous studies [14,29,30], we also showed that miR-29s inhibit cell proliferation and induce apoptosis in NSCLC cells. A previous study showed that miR-29s sensitize tumor cell to apoptosis by targeting Mcl-1 or the p53 pathway [29,30]. Our results showed that WIF-1 knockdown abolishes these effects of miR-29s, suggesting that inhibition of Wnt signaling could be another potential mechanism mediating the anti-tumor effects of miR-29s.

Desjobert et al. showed that the expression of miR-29a was regulated by methylation and suggested the existence of a positive feedback loop involving DNMT3b [17]. Our data extend these observations and show that miR-29b expression is upregulated in response to treatment with a demethylating agent, which supports the presence of a positive feedback loop in NSCLC cells. However, a similar regulation of expression by methylation was not observed for miR-29c. This could be attributed to the fact that miR-29c and miR-29b2 is expressed from a different locus (Chr 1q32.2) and the regulation pattern may be different from that of miR-29a and miR-29b1 on chromosome 7. In addition, a different positive feedback loop regulating the expression of miR-29s via c-Myc has been proposed. The effect of the c-Myc oncogene on the repression of mir-29 promoter activity has been shown previously [16,31]. Furthermore, this occurs downstream of the Wnt pathway and could be activated by β-catenin. Therefore, miR-29s may indirectly suppress the transcription of c-Myc by modulating the Wnt/β-catenin signaling pathway.

In summary, we showed that DNMT3A and DNMT3B are involved in the down-regulation of WIF-1 expression and that miR-29s restore WIF-1 expression by suppressing DNMT3A and DNMT3B. Enforced expression of these miRNAs in lung cancer cells blocks Wnt/β-catenin signaling and inhibits tumor growth. In addition, the expression of miR-29a and miR-29b may be partly

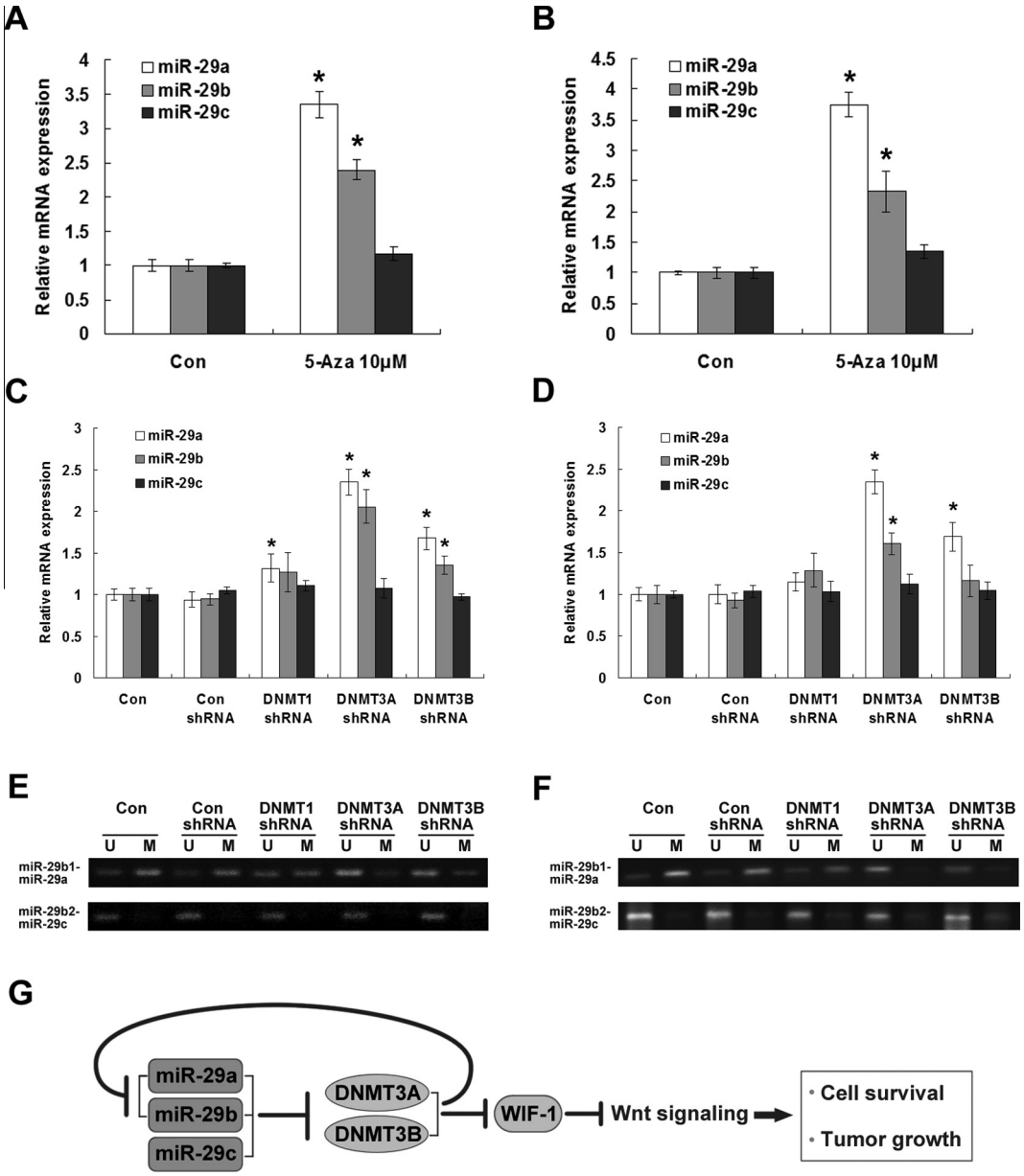

Fig. 4. DNMT3A and DNMT3B catalyze the methylation of miR-29a and miR-29b. (A and B) The expression of miR-29 family members was assessed by real-time PCR in A549 (A) and H1299 (B) cells treated with the demethylating agent 5-Aza-deoxycytidine. (C and D) shRNA mediated knockdown of DNMT3A and DNMT3B restores the expression of miR-29a and miR-29b in A549 (C) and H1299 (D) cells. Suppressions of DNMT3A and DNMT3B inhibited the methylation of the miR-29b1-miR-29a promoter in A549 (E) and H1299 (F) cells. (G) Schematic illustration of the regulation of the Wnt/β-catenin signaling pathway by miR-29s. Suppression of the expression of DNMT3A and DNMT3B by miR-29s reduces the methylation of miR-29s themselves and WIF-1 gene methylation. Restoration of WIF-1 expression inhibits the activity of the Wnt/β-catenin signaling pathway and tumor growth. $^{*}p < 0.05$ compared with control (Con).

regulated by DNMT3A and DNMT3B in a positive feedback loop. Our study provides new insights into the involvement of miRNAs in the Wnt pathway and underscores the fundamental role of these miRNAs as tumor suppressor genes.

References

[1] P. Guo, Z.L. Huang, P. Yu, K. Li, Trends in cancer mortality in China: an update, Ann. Oncol. 23 (10) (2012) 2755–2762.

[2] P. Polakis, Wnt signaling and cancer, Genes Dev 14 (15) (2000) 1837–1851.

[3] K. Uematsu, B. He, L. You, Z. Xu, F. McCormick, et al., Activation of the Wnt pathway in non small cell lung cancer: evidence of dishevelled overexpression, Oncogene 22 (46) (2003) 7218–7221.

[4] B.T. MacDonald, K. Tamai, X. He, Wnt/beta-catenin signaling: components, mechanisms, and diseases, Dev. Cell 17 (1) (2009) 9–26.

[5] M.T. Veeman, J.D. Axelrod, R.T. Moon, A second canon: functions and mechanisms of beta-catenin-independent Wnt signaling, Dev. Cell 5 (3) (2003) 367–377.

[6] X. Xu, P.L. Sun, J.Z. Li, S. Jheon, C.T. Lee, et al., Aberrant Wnt1/β-catenin expression is an independent poor prognostic marker of non-small cell lung cancer after surgery, J. Thorac. Oncol. 6 (4) (2011) 716–724, http://dx.doi.org/10.1097/JTO.0b013e31820c5189.

[7] J. Mazieres, B. He, L. You, Z. Xu, A.Y. Lee, et al., Wnt inhibitory factor-1 is silenced by promoter hypermethylation in human lung cancer, Cancer Res. 64 (14) (2004) 4717–4720.

[8] A. Jeltsch, Beyond Watson and Crick: DNA methylation and molecular enzymology of DNA methyltransferases, ChemBioChem 3 (4) (2002) 274–293.

[9] I. Girault, S. Tozlu, R. Lidereau, I. Bièche, Expression analysis of DNA methyltransferases 1, 3A, and 3B in sporadic breast carcinomas, Clin. Cancer Res. 9 (12) (2003) 4415–4422.

[10] Y. Saito, Y. Kanai, T. Nakagawa, M. Sakamoto, H. Saito, et al., Increased protein expression of DNA methyltransferase (DNMT) 1 is significantly correlated with the malignant potential and poor prognosis of human hepatocellular carcinomas, Int. J. Cancer 105 (4) (2003) 527–532.

[11] H. Kim, Y.M. Kwon, J.S. Kim, J. Han, Y.M. Shim, et al., Elevated mRNA levels of DNA methyltransferase-1 as an independent prognostic factor in primary nonsmall cell lung cancer, Cancer 107 (5) (2006) 1042–1049.
[12] R.K. Lin, H.S. Hsu, J.W. Chang, C.Y. Chen, J.T. Chen, et al., Alteration of DNA methyltransferases contributes to 5′CpG methylation and poor prognosis in lung cancer, Lung Cancer 55 (2) (2007) 205–213.
[13] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2) (2004) 281–297.
[14] M. Fabbri, R. Garzon, A. Cimmino, Z. Liu, N. Zanesi, et al., MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B, Proc. Natl. Acad. Sci. USA 104 (40) (2007) 15805–15810.
[15] S. Volinia, G.A. Calin, C.G. Liu, S. Ambs, A. Cimmino, et al., A microRNA expression signature of human solid tumors defines cancer gene targets, Proc. Natl. Acad. Sci. USA 103 (7) (2006) 2257–2261.
[16] J.L. Mott, S. Kurita, S.C. Cazanave, S.F. Bronk, N.W. Werneburg, et al., Transcriptional suppression of mir-29b-1/mir-29a promoter by c-Myc, hedgehog, and NF-kappaB, J. Cell. Biochem. 110 (5) (2010) 1155–1164.
[17] C. Desjobert, M.H. Renalier, J. Bergalet, E. Dejean, N. Joseph, et al., MiR-29a down-regulation in ALK-positive anaplastic large cell lymphomas contributes to apoptosis blockade through MCL-1 overexpression, Blood 117 (24) (2011) 6627–6637.
[18] C.F. Mountain, Revisions in the international system for staging lung cancer, Chest 111 (6) (1997) 1710–1717.
[19] L. You, B. He, Z. Xu, K. Uematsu, J. Mazieres, et al., Inhibition of Wnt-2-mediated signaling induces programmed cell death in non-small-cell lung cancer cells, Oncogene 23 (36) (2004) 6170–6174.
[20] B. He, L. You, K. Uematsu, Z. Xu, A.Y. Lee, et al., A monoclonal antibody against Wnt-1 induces apoptosis in human cancer cells, Neoplasia 6 (1) (2004) 7–14.
[21] F. Ugolini, E. Charafe-Jauffret, V.J. Bardou, J. Geneix, J. Adélaïde, et al., WNT pathway and mammary carcinogenesis: loss of expression of candidate tumor suppressor gene SFRP1 in most invasive carcinomas except of the medullary type, Oncogene 20 (41) (2001) 5810–5817.
[22] H. Suzuki, D.N. Watkins, K.W. Jair, K.E. Schuebel, S.D. Markowitz, et al., Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer, Nat. Genet. 36 (4) (2004) 417–422.
[23] H. Taniguchi, H. Yamamoto, T. Hirata, N. Miyamoto, M. Oki, et al., Frequent epigenetic inactivation of Wnt inhibitory factor-1 in human gastrointestinal cancers, Oncogene 24 (53) (2005) 7946–7952.
[24] Y. Deng, B. Yu, Q. Cheng, J. Jin, H. You, et al., Epigenetic silencing of WIF-1 in hepatocellular carcinomas, J. Cancer Res. Clin. Oncol. 136 (8) (2010) 1161–1167.
[25] M. Suzuki, H. Shigematsu, T. Nakajima, R. Kubo, S. Motohashi, et al., Synchronous alterations of Wnt and epidermal growth factor receptor signaling pathways through aberrant methylation and mutation in non-small cell lung cancer, Clin. Cancer Res. 13 (20) (2007) 6087–6092.
[26] M. Cebrat, L. Strzadala, P. Kisielow, Wnt inhibitory factor-1: a candidate for a new player in tumorigenesis of intestinal epithelial cells, Cancer Lett. 206 (1) (2004) 107–113.
[27] J.L. Mott, S. Kobayashi, S.F. Bronk, G.J. Gores, Mir-29 regulates Mcl-1 protein expression and apoptosis, Oncogene 26 (42) (2007) 6133–6140.
[28] R. Garzon, C.E.A. Heaphy, V. Havelange, M. Fabbri, S. Volinia, et al., MicroRNA 29b functions in acute myeloid leukemia, Blood 114 (26) (2009) 5331–5341.
[29] Y. Xiong, J.H. Fang, J.P. Yun, J. Yang, Y. Zhang, et al., Effects of MicroRNA-29 on apoptosis, tumorigenicity, and prognosis of hepatocellular carcinoma, Hepatology 51 (3) (2010) 836–845.
[30] S.Y. Park, J.H. Lee, M. Ha, J.W. Nam, V.N. Kim, MiR-29 miRNAs activate p53 by targeting p85[alpha] and CDC42, Nat. Struct. Mol. Biol. 16 (1) (2009) 23–29.
[31] T.C. Chang, D. Yu, Y.S. Lee, E.A. Wentzel, D.E. Arking, et al., Widespread microRNA repression by Myc contributes to tumorigenesis, Nat. Genet. 40 (1) (2008) 43–50.