SPAG6 regulates cell apoptosis through the TRAIL signal pathway in myelodysplastic syndromes

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Abstract. Myelodysplastic syndromes (MDSs) are a group of malignant clone hematopoietic stem-cell diseases, and the evolution and progression of MDS depend on the abnormal apoptosis of bone marrow cells. Our previous studies have indicated that sperm-associated antigen 6 (SPAG6), located in the uniparental disomy regions of myeloid cells, is over-expressed in patients with MDS as compared to controls, and SPAG6 can inhibit apoptosis of SKM-1. However, the concrete mechanism is still unclear. In the present study, it was found that the TNF-related apoptosis-inducing ligand (TRAIL) signal pathway was activated when the expression of SPAG6 was inhibited by SPAG6-shRNA lentivirus in SKM-1 cells. Additionally, the results of flow cytometry, Cell Counting Kit-8 assay and western blot analysis implied that the TRAIL signal pathway could be inhibited by a high expression of SPAG6. However, SPAG6 cannot influence the expression of TRAIL death receptors, except for FADD. Additionally the interaction between FADD and TRAIL death receptors also increased in SKM-1 cells infected with SPAG6-shRNA lentivirus. Thus, our study demonstrates that SPAG6 may regulate apoptosis in SKM-1 through the TRAIL signal pathway, indicating that SPAG6 could be a potential therapeutic target.

Introduction

Myelodysplastic syndromes (MDSs), one of the five major categories of myeloid neoplasms, are defined as clonal stem-cell disorders characterized by ineffective hematopoiesis in one or more of the lineages of the bone marrow, and the progression is from blood cytopaenias to acute myeloid leukaemia (AML) (1). AML, which is the most common type of acute leukemia in adults, is fatal in more than 50% of patients that do not receive current treatments (2), and the mortality of MDS is primarily caused by pancytopenia or transformation to AML. Therefore, the discovery of the molecular mechanisms would be significant for better prognoses. It has been discovered that MDS are one of the clonal haemopathies, in that an aberration within a haematopoietic progenitor cell gives rise to the entire disease (3). Additionally, some abnormal karyotypes have been found in the patients with MDS, including del (5q), monosomy 7 or del (7q), trisomy 8 and del (20q), whereas ~50% patients have a normal karyotype (4-6).

The pathogenesis of MDS has not yet been completely elucidated. Recently, uniparental disomy (UPD) has garnered attention, and some studies have indicated that UPD is related to hematologic malignancies including both MDS and AML (7,8). Furthermore, sperm-associated antigen 6 (SPAG6) was shown to be overexpressed in some patients with MDS in a genome wide single nucleotide polymorphism analysis, which implies that SPAG6 may play a role in the pathogenesis of MDS (9).

SPAG6, which is the orthologue of *Chlamydomonas* PF16, is essential for sperm flagellar motility and maintenance of the structural integrity of mature sperm (10). In the last decade, several research groups have focused on the relationship between SPAG6 and malignancies. Seven genes, including WT1 and SPAG6, were found to be vastly overexpressed in patients with AML as compared to healthy bone marrow via genome-wide expression profiling (11). Furthermore, SPAG6 is highly expressed at the break point of t(10;11)(p12;q14) in patients with CALM/AF10-positive leukemias (12). In our previous study, when the expression of SPAG6 was silenced by SPAG6-shRNA lentivirus, the growth of SKM-1 and K652 was markedly inhibited, and apoptosis was increased (13). The above evidence indicates that SPAG6 may promote cellular growth by preventing apoptosis. However, the specific mechanism of anti-apoptosis is still unknown.

Several studies imply that abnormal apoptosis is one of the underlying mechanisms in MDS, and tumor necrosis factor (TNF)-α, Fas ligand, TNF-related apoptosis-inducing ligand (TRAIL) and other pro-apoptotic cytokines may be the major factors (14). In the last decades, the role of TRAIL in the pathogenesis of malignancies has been observed. TRAIL, also known as Apo2 ligand (Apo2L), is a member of the TNF ligand superfamily, and it is induced in tumor cells, but seldom

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in normal cells (15). Recently, two researchers explored the possible potential phenotype related to the TRAIL signal pathway using a genome-wide siRNA screen, and it was found that SPAG6 and this pathway may have a correlation, which was recorded in the GenomeRNAi-database (http://www.genomernai.org) (16).

The aim of this study is to clarify the regulatory mechanism of SPAG6 in cell apoptosis of MDS by TRAIL signal pathway, which may provide a new perspective in the development of MDS.

Materials and methods

Cell culture and infection. The human MDS cell line SKM-1, was kindly provided by Professor Zhou at the Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). The erythroleukemia K562 and the histiocytic lymphoma U937 cell lines were kept frozen in our laboratory. Cell line SKM-1, U937 and K562 were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS; Capricorn Scientific GmbH, Ebsdorfergrund, Germany) and no antibiotic in 5% CO_2, 95% air incubator at 37˚C. For the infection protocol, SKM-1 cells at the exponential stage were plated in 6-well plates ($5x10^4$ cells/well), and were infected with the SPAG6-shRNA lentivirus or NC-shRNA lentivirus at a multiplicity of infection (MOI) of 20 in the concentration of 5 μg/ml polybrene, respectively. After 10 h, the cells were washed and then resuspended in complete medium. After 5 days, the transfection efficiencies were evaluated by flow cytometry.

Cell treatment. SKM-1, K562 and U937 were seeded in 6-well plates (1×10^5), and were treated with recombinant human sTRAIL (310-04; Peprotech, Rocky Hill, NJ, USA), respectively. The treated concentrations were as follows: 0, 20, 40, 60, 80, 100 and 50 ng/ml. After 24 h, the cells were collected, and the apoptosis rates were measured by flow cytometry.

Flow cytometry. The apoptosis rates were detected using Annexin V and 7-ADD double-staining by flow cytometry. Following the cell treatments, the cells were collected and washed twice with phosphate-buffered saline (PBS). The early apoptotic cells contained Annexin V^+/PI^-, while the late apoptotic cells contained Annexin V^+/PI^+. Both of the two stages of apoptotic cells were counted, and the results were illustrated as a percentage of the total cell count. The techniques were supported by the Life Science Department of Chongqing Medical University (Chongqing, China).

RNA isolation and real-time PCR. The total cellular RNA from each group was extracted using the TRIzol reagent, and the reverse transcription reaction was performed using the Prime Script™ RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). The cDNA was amplified in a 10-μl PCR mix with 5 μl of SYBR-Green super mixture. The PCR reactions were performed in a CFX-Connect Real-Time PCR system (Bio-Rad Laboratories Inc., Hercules, CA, USA). The cycling parameters were: 95˚C for 30 sec, then 40 cycles at 95˚C for 5 sec and 60˚C for 30 sec. PCR primers were as follows: SPAG6 forward, 5'-CCTTTCAGCTCTCAGTCAGGTTTC-3' and reverse, 5'-TCTTCACGTTTCATCCTTGTCCTT-3'; death receptor 4 (DR4) forward, 5'-TCGCTGTCCACTTTCGTCT-3' and reverse, 5'-GGCGTTCCGTCCAGTTTTG-3'; DR5 forward, 5'-AAGACCCTTGTGCTCGTTGT-3' and reverse, 5'-GCTGCAACTGTGACTCCTAT-3'; and GAPDH forward, 5'-CTTTGGTATCGTGGAAGGACTC-3' and reverse, 5'-GTAGAGGCAGGGATGATGTTCT-3'. The PCR reactions were performed in a CFX-Connect Real-Time PCR system for 40 cycles (Bio-Rad Laboratories, Inc.). The relative gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method.

Protein isolation and western blot analysis. The cells were collected and lysed in RIPA lysis buffer (Beyotime, Beijing, China) containing 1 μM PMSF, and the concentration was measured using the BCA Protein Assay kit (Beyotime). The total cell lysate was denatured via boiling and a total of 50 μg of protein per lane was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to PVDF membranes. The membranes were blocked with 5% bovine serum albumin for 90 min at room temperature and then incubated overnight at 4˚C with specific antibodies. The primary antibodies were as follows: GAPDH (AG019, 1:1,000; Beyotime) cleaved caspase-8 (9496, 1:500; Cell Signaling Technology, Inc., Beverly, MA, USA); SPAG6, PARP, cleaved PARP, caspase-8, DR4, DR5 and FADD (ab155653, ab32138, ab32561, ab32397, ab8414, ab181846, ab108601, 1:1,000; all from Abcam, Cambridge, UK); caspase-3 and BID (YT0656, YT0488, 1:500; both from Immunoway, Newark, DE, USA); and BAX and BAK (RLT0456, RLT0449, 1:500; both from Ruiying Biological, Suzhou, China). After five washes with TBS-Tween-20, the membranes were incubated with a goat anti-rabbit of goat anti-mouse peroxidase-conjugated second antibody (A0216, A0208, 1:1,000; Beyotime) for 1 h at 37˚C. Then, the excess antibody was removed from the blots with TBS-Tween-20 three times before incubation in ECL. The protein expression levels were analysed using Vilber Fusion software (Fusion FX5 Spectra; Vilber Lourmat, Marne-La-Vallée, France).

The Cell Counting Kit-8 (CCK-8) assay. Apoptosis was measured by a CCK-8 assay. Cells in exponential growth were seeded in 96-well plates at a density of $8x10^3$ cells/well, and then, they were treated with recombinant human sTRAIL (rTRAIL) containing different concentrations for 24 h, respectively. Finally, 10 μl of CCK-8 (Dojindo Laboratories, Kumamoto, Japan) solution was added to each well, and the plates were incubated at 37˚C for 100 min. Cell apoptosis was estimated by measuring the absorbance at 450 nm using the plate reader. All conditions were tested in five replicates.

Immunoprecipitation. The cells infected with the lentivirus were collected after infection and lysed in RIPA lysis buffer. The solution was centrifuged at 12,000 rpm for 10 min at 4˚C and the supernatants were kept. Protein A agarose and antibody (ab108601, 1:100, ab14738, ab181846, 1:2,000; Abcam) diluted with RIPA buffer were mixed and incubated overnight at 4˚C. Then the total protein was mixed and incubated overnight at 4˚C. After incubation, the mixture was washed six

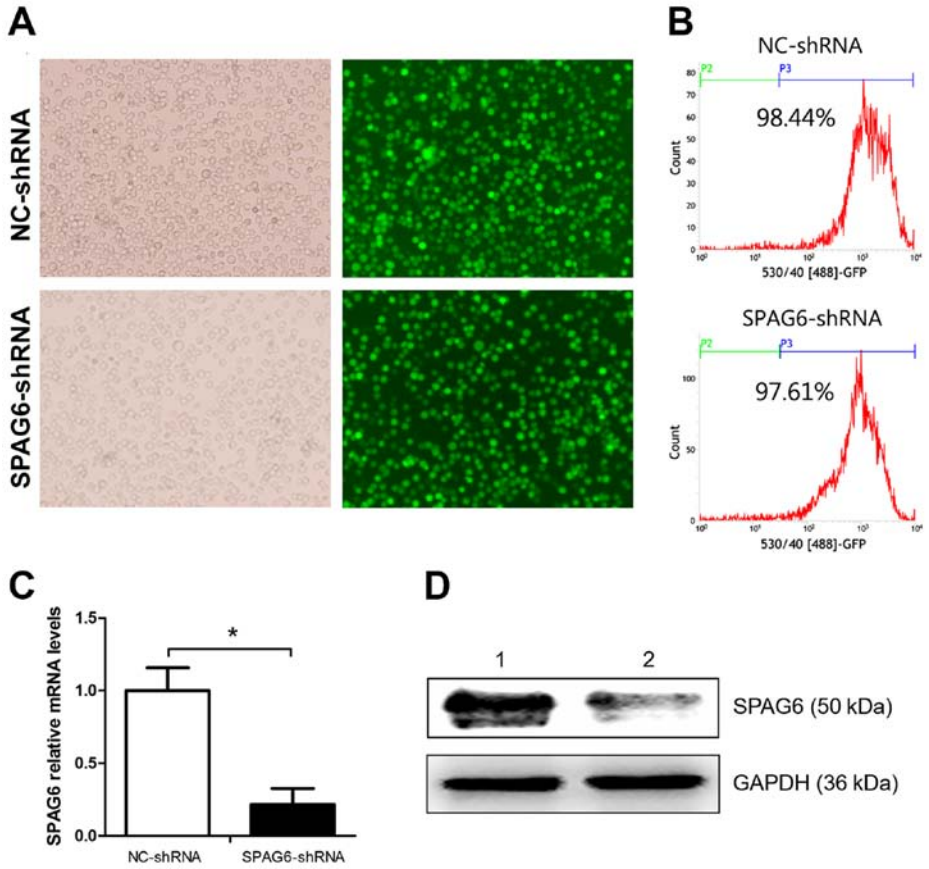

Figure 1. Knockdown of SPAG6 expression by lentivirus. (A) SKM-1 cells infected with lentivirus were observed under a fluorescence microscope (x40 magnification). (B) Transfection efficiencies were detected by flow cytometry. (C) SPAG6 expression was inhibited at the mRNA level. (D) Protein expression of SPAG6 was measured by western blot analysis, and GAPDH was used as the internal reference. *P<0.05. Lane 1, NC-shRNA group; lane 2, SPAG6-shRNA group. SPAG6, sperm-associated antigen 6.

times with different types of washing buffer. Finally, a western blot analysis was performed for immunoblot analysis.

Statistical analysis. All the results were technically repeated three times, and were expressed as the mean ± standard deviation (SD). The significant differences between groups were assessed by one-way analysis of variance (ANOVA) and Student's t-test (SPSS version 20.0; SPSS, Inc., Chicago, IL, USA) and a value of P<0.05 was considered statistically significant.

Results

Lentivirus-mediated SPAG6 silence in SKM-1 cells. The lentivirus expressing shRNA was used against SPAG6 for gene silencing in SKM-1 cells, and the SKM-1 cells infected with NC-shRNA lentivirus were used as a control group. Six days after infection, abundance of cells with green fluorescence could be observed under a fluorescence microscope (Fig. 1A). The transfection efficiencies were detected by FACS, and the results were 97.19±3.21% and 88.31±12.81% (Fig. 1B). This demonstrated that the SKM-1 cells were successfully infected by the lentivirus. The inhibitory degree of SPAG6 was measured by qRT-PCR and western blot analysis. The results showed that both the mRNA and protein levels were reduced in cells infected with SPAG6 shRNA-lentivirus as compared to the control group (Fig. 1C and D).

Reducing the expression of SPAG6 activates the TRAIL signal pathway. Apoptosis was analysed by Annexin V and 7-AAD assays, as illustrated in Fig. 2A and B, and the apoptosis rates in SKM-1 infected with SPAG6-shRNA were significantly higher than those in the control group (NC-shRNA, 8.65±2.07% vs. SPAG6-shRNA, 20.20±2.11%; P<0.05). To explore the activation of the TRAIL signal pathway, the expression of related proteins was examined. Western blot analysis revealed that the expression levels of cleaved PARP, caspase-8 and cleaved caspase-8 were obviously higher in SKM-1 infected with SPAG6-shRNA lentivirus than in the control group, whereas the expression of PARP showed no significant difference between the two groups (Fig. 2C and D). Furthermore, the expression of apoptotic factors, including BAK, BAX, BID and caspase-3, also showed statistical differences in the two groups (Fig. 3E and F). These data indicate that the TRAIL signal pathway was activated when the expression of SPAG6 was reduced.

SPAG6 resists apoptosis induced by TRAIL. Following rTRAIL treatment, the percentage of apoptosis cells increased, and the flow cytometry data indicated that the higher concentration of TRAIL led to higher apoptosis rates (Fig. 3A and B). To investigate the growth of SKM-1 cells treated with different concentrations of TRAIL, CCK-8 assays were performed to analyse cell proliferation. Cell proliferation was suppressed in the cells treated with TRAIL as compared to the normal

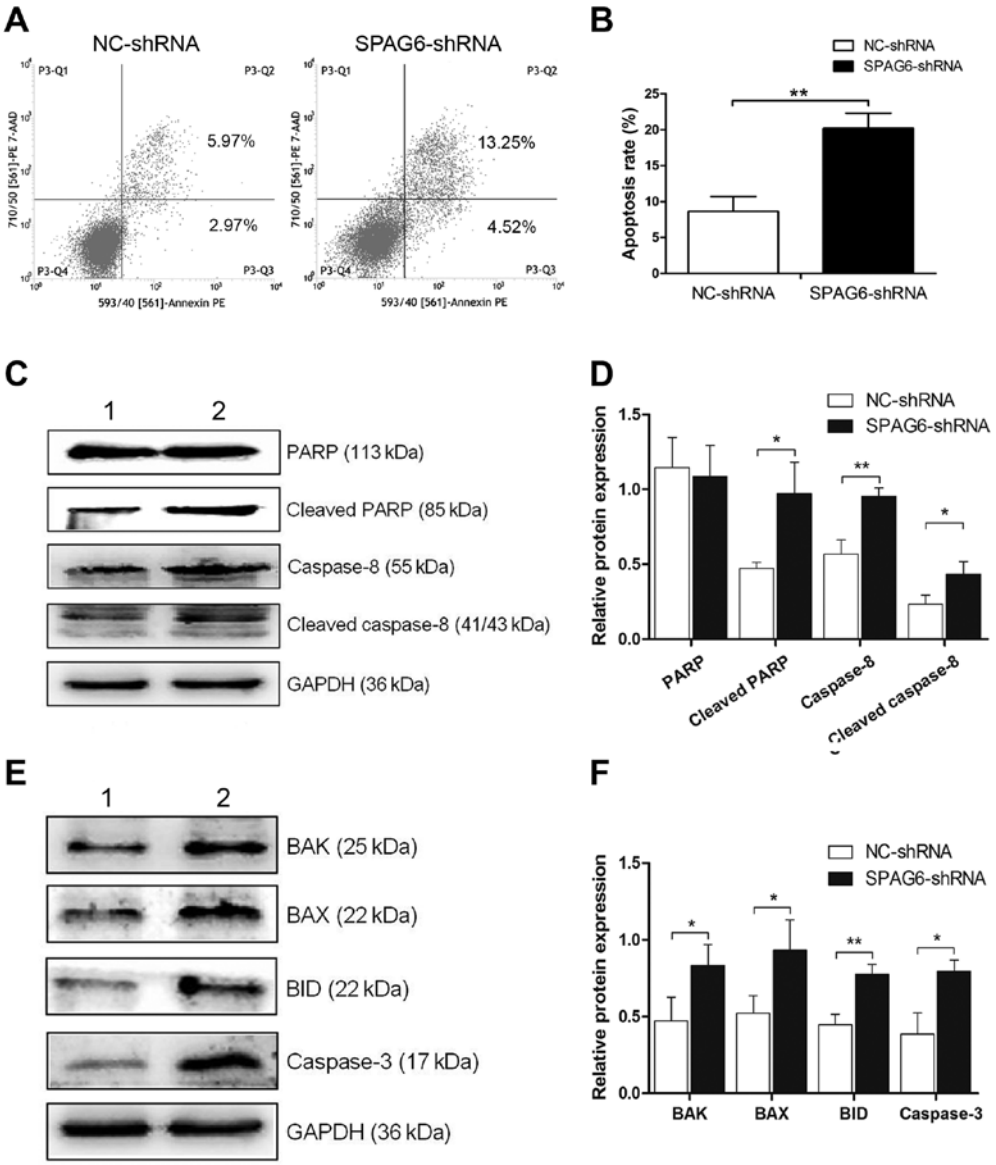

Figure 2. TRAIL signal pathway is activated when the expression of SPAG6 is inhibited. (A) After transfection with lentivirus, the apoptosis rate was evaluated by flow cytometry. (B) The analysis of the apoptosis rates is shown in (A). The apoptosis rate of SKM-1 cells infected with SPAG6-shRNA significantly increased as compared to cells infected with NC-shRNA. (C) Expression of PARP, cleaved PARP, caspase-8 and cleaved caspase-8 protein was detected by western blot analysis. (D) TRAIL signal pathway was activated. (E) Expression of BAK, BAX, BID and caspase-3 protein was detected by western blot analysis. (F) These proteins showed statistical differences between the groups. The data are shown as mean ± SD. *P<0.05, **P<0.01. Lane 1, NC-shRNA group; lane 2, SPAG6-shRNA group. TRAIL, TNF-related apoptosis-inducing ligand; SPAG6, sperm-associated antigen 6.

control group (Fig. 3C). In addition, western blot analysis showed that the expression of related proteins was higher after treatment (Fig. 3D and E). However, as shown in Fig. 3F and G, in the SKM-1, U937 and K562 cells after treatment with the same concentration of TRAIL, the apoptosis rates were not significantly different except for that of SKM-1 (the expression of SPAG6, SKM-1<U937<K562), and similar results were also observed in the CCK-8 assays (Fig. 3H). These data illustrate that a high expression of SPAG6 may resist apoptosis induced by TRAIL.

SPAG6 has no effect on the expression of TRAIL death receptors, except for FADD. TRAIL induces apoptosis through DR4 (TNFRSF10A) and DR5 (TNFRSF10B). Therefore, the degree of apoptosis induced by TRAIL is closely related to the levels of DR4 and DR5. As illustrated in the figure, both the mRNA and protein levels of the two receptors were not changed (Fig. 4A-C). However, the expression of FADD was statistically different (Fig. 4B and C), indicating that the regulation of apoptosis may not be through DR4 and DR5 expression.

SPAG6 influences the interaction between the FADD and TRAIL death receptors. As shown in the Fig. 4, the expressions of DR4 and DR5 showed no difference. Therefore, immunoprecipitation was used to explore the relationship between the FADD and TRAIL death receptors. FADD was used as the bait protein to immunoprecipitate DR4 and DR5. Then, DR4 and DR5 were blotted. As shown in the figure, the interaction between FADD and both death receptors increased in SKM-1 infected with SPAG6 shRNA-lentivirus as compared to the control group (Fig. 4D).

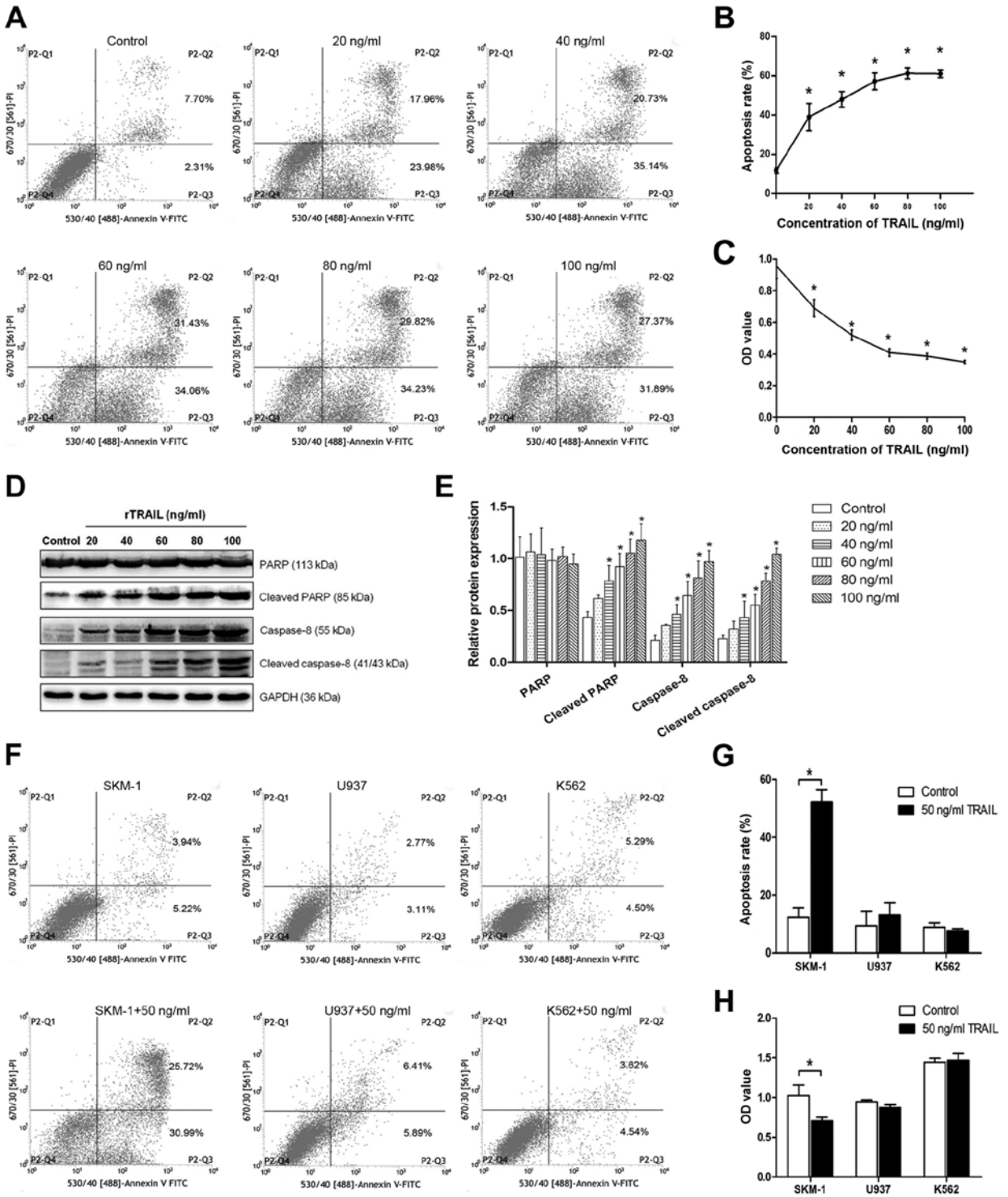

Figure 3. SPAG6 may resist apoptosis induced by TRAIL. (A) Following rTRAIL treatment, the apoptosis rates of SKM-1 cells were detected by flow cytometry. (B) A higher concentration of rTRAIL led to higher apoptosis rates in SKM-1 cells. (C) The proliferation was assessed by a CCK-8 assay. (D) PARP, cleaved PARP, caspase-8 and cleaved caspase-8 protein expression were detected by western blot analysis. (E) The analysis of the relative protein expression. (F) After SKM-1, U937 and K562 cells were treated with the same concentration of TRAIL, the apoptosis rates were measured by flow cytometry. (G) The apoptosis rates were not significantly different in the U937 and K562 cells (the expression of SPAG6, SKM-1<U937<K562; https://portals.broadinstitute.org/ccle/home). (H) The proliferation showed the same results as the apoptosis rates. The data are shown as mean ± SD. *P<0.05. SPAG6, sperm-associated antigen 6; TRAIL, TNF-related apoptosis-inducing ligand.

Discussion

Apoptosis is a process of programmed cell death, which can occur in multicellular organisms in order to maintain homeostasis. In brief, apoptosis is initiated through two pathways, the extrinsic pathway and the intrinsic pathway mediated by mitochondrion (17). Abnormal apoptosis, including resistance to apoptosis, is regarded as an important mechanism of tumorigenesis (18). MDS is characterized by ineffective hematopoiesis leading to peripheral blood

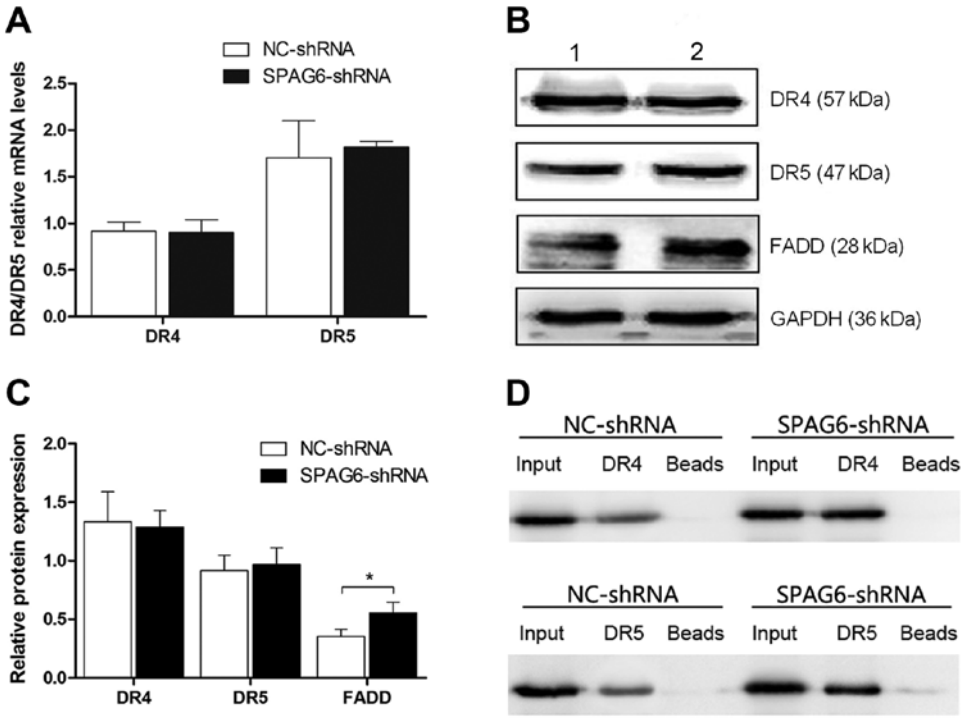

Figure 4. SPAG6 can influence the interaction between FADD and TRAIL death receptors. (A) The mRNA expression of DR4 and DR5 showed no statistical difference. (B) The DR4, DR5 and FADD protein expression were determined by western blot analysis. (C) SPAG6 showed no effect on the expression of TRAIL death receptors, except for FADD. (D) Immunoprecipitation was used to explore the interaction between FADD and TRAIL death receptors. The interaction between FADD and death receptors increased in SKM-1 cells infected with SPAG6-shRNA lentivirus. The data are shown as mean ± SD. *P<0.05. Lane 1, NC-shRNA group; lane 2, SPAG6-shRNA group. SPAG6, sperm-associated antigen 6; TRAIL, TNF-related apoptosis-inducing ligand.

cytopenia, and one of the possible mechanisms is excessive apoptosis in hematopoietic precursors. It has been found that TRAIL and its receptors are expressed at a low level in normal marrow, and the opposite occurs in MDS marrow cells (19). Furthermore, the expression of TRAIL signal inhibitor including cFLIP, XIAP and the Bcl-2 family, is higher in patients with MDS in advanced stages than those with MDS in the early stages (20-22). The evidence indicates that the TRAIL signal pathway may play a role in the development of MDS.

SPAG6 was first detected in human testis tissue. Its major functions are participation in the maturation of reproductive cells and maintaining sperm motility and fertility (10,23,24). Recent studies have found that SPAG6 is upregulated in CALM/AF10-positive leukemia and pediatric AML (11,12). Additionally, a prospective multicenter study indicated that patients with AML may obtain a better prognosis and relapse-free survival (RFS) when SPAG6 is expressed at a low level (25). Furthermore, in lung and breast cancer, SPAG6 is defined as a novel cancer-testis (CT) antigen, which can be considered as a tumor marker and potential candidate for use in cancer therapy (26), implying that SPAG6 may lead to carcinogenesis and can be a parameter for assessing their efficacy or prognosis. However, the molecular mechanism of SPAG6 in hematologic malignancy has seldom been studied.

Therefore, in the present study, we explored the relationship between SPAG6 and the TRAIL signal pathway to illustrate the possible mechanism of the regulation of apoptosis in SKM-1. TRAIL can induce apoptosis in cells through its receptors at the cell surface, DR4 and DR5 (27). After TRAIL binds to receptors, the adapter protein FADD is recruited through cytoplasmic death domains, and then, FADD interacts with pro-caspase-8 recruited to form the death-inducing signaling complex (DISC). DISC then mediates the activation of pro-caspase-8 (28). Caspase-8 directly activates caspase-3 to cleave the substrates, such as PARP (29). Therefore, the activation of caspase-8 and the cleavage of PARP are chosen as parameters to measure apoptosis. Moreover, BID, a target of active caspase-8, is cleaved to form truncated BID (tBID) and tBID then leads the participation of mitochondrial apoptotic factors (30). As is shown in the results, after the expression of SPAG6 was reduced by the lentivirus, the apoptosis rates increased, and the TRAIL signal pathway was activated, and both the intrinsic and extrinsic pathways were triggered. This results suggests that SPAG6 may affect apoptosis through the TRAIL signaling pathway.

A preliminary study indicated that a high expression of SPAG6 is connected with apoptosis resistance in SKM-1 and K562 (13). In this study, when SKM-1 cells were treated with different concentrations of rTRAIL, the apoptosis rates showed an increased tendency. However, given the same concentration of rTRAIL, cells with high expression levels of SPAG6 exhibited insensitivity to apoptosis induced by rTRAIL. In addition, the expression of DR4 and DR5 showed no differences at the mRNA and protein levels, but the expression of FADD increased. These data imply that the regulation may not occur through the receptors. In order to explore the possible mechanism, immunoprecipitation was used to measure the interaction between FADD and both DR4 and DR5. It was found that the interaction between FADD and DR4 and that

between FADD and DR5 both increased. Moreover, p53 is the most commonly mutated gene in human cancer (31), and large quantities of studies have demonstrated its anticancer functions. Additionally, some studies have shown that p53 may also participate in the regulation of apoptosis induced by TRAIL, and the possible approaches are to influence the expression of the TRAIL receptors (32,33) or the BCL-2 family (34). In addition, TRAIL can induce not only the initiation of apoptosis but also the non-apoptotic pathways, such as nuclear factor-κB (NF-κB), phosphatidylinositol 3-kinase (PI3K) and Akt, and mitogen activated protein kinases (MAPKs) (35). It is important to note that these studies are performed on the cellular level, so an *in vivo* study is needed to confirm the results. Furthermore, there are limitations inherent in using a cell line in culture versus using primary bone marrow cells from patients. Therefore, further studies are needed to explore the possible mechanisms.

In conclusion, this study demonstrates that SPAG6 may regulate apoptosis through the TRAIL signal pathway by inhibiting the expression of FADD and the interactions between FADD and the TRAIL death receptors. TRAIL has been proven to possess anticancer functions. In animal models, it has shown that the growth of a TRAIL-sensitive tumor is suppressed by rTRAIL without significant system toxicity (36,37). MDS treatments have rapidly improved; however, there is still lack of effective methods, and antileukemic drugs can enhance apoptosis mediated by TRAIL (38). The above may indicate a new therapy for MDS. Therefore, SPAG6 may be a potential target in therapy for MDS, and it may also be worthwhile to explore drugs against SPAG6 function.

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