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Original Article

lncRNA PCAT-1 interacting with FZD6 contributes to the malignancy of acute myeloid leukemia cells through activating Wnt/β-catenin signaling pathway

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Abstract: Accumulating evidence has suggested the involvement of long noncoding RNAs (lncRNAs) on the acute myeloid leukemia (AML). Therefore, this study aimed to investigate the unknown function of lncRNA Prostate cancer-associated transcript-1 (PCAT-1) in AML cells. Our data found that PCAT-1 was highly expressed in AML-M1/2 and AML-M3 patients than normal controls and its expression was significantly up-regulated in AML cell lines Kasumi-6 and HL-60. The functional experiments demonstrated that knockdown of PCAT-1 remarkably inhibited proliferation, arrested cell cycle progression and triggered apoptosis of AML cells. Mechanistically, we revealed that PCAT-1 could directly interact with FZD6 protein to regulate its stability. Overexpression of FZD6 partly abolished the effects of PCAT-1 silencing on AML cells. Our integrated experiments then suggested that PCAT-1 could activate the Wnt/β-catenin signaling pathway in an FZD6-dependent manner. Taken together, the present study indicated that PCAT-1 interacting with FZD6 to activate Wnt/β-catenin signaling, which may play an important role in the pathogenesis of AML.

Keywords: Acute myeloid leukemia, PCAT-1, FZD6, Wnt/β-catenin signaling

Introduction

Acute myeloid leukemia (AML) is a cancer of the myeloid line of blood cells that infiltrate the bone marrow, blood, and other tissues. It is a result of a number of genetic abnormalities including mutations and chromosomal rearrangements [1]. Therefore, a deeper understanding of the molecular mechanisms becomes essential for finding novel therapeutic targets against AML.

Long non-coding RNAs (lncRNAs) are defined as non-protein coding transcripts longer than 200 nt and emerging evidence demonstrated that lncRNAs play a critical role in regulating pathological and physiological processes of various cancers, including lung cancer [2], breast cancer [3], multiple myeloma [4] and AML [5, 6]. lncRNA SBF2-AS1 was increased in AML and knockdown of SBF2-AS1 inhibited cells proliferation and induced apoptosis in AML cells [7]. In addition, ANRIL was found to promote malignant cell survival and cell glucose metabolism to accelerate AML progression via AMPK/SIRT1 signaling pathway [8]. However, up to date, still limited lncRNAs are identified in AML. lncRNA Prostate cancer-associated transcript-1 (PCAT-1) is a novel lncRNA which was originally discovered in prostate cancer [9]. Currently, plenty of studies identified that PCAT-1 is highly expressed in numerous cancer and elevated PCAT-1 is correlated with poor overall survival [10]. Nevertheless, the expression profile of PCAT-1 and its role in AML is completely unknown.

Here, we found that PCAT-1 was upregulated in the AML patients and AML cell lines. Knockdown of PCAT-1 caused significant inhibition of cell proliferation, induced the cycle arrest and triggered apoptosis of AML cells. Moreover, we revealed that PCAT-1 directly bound to frizzled class receptor 6 (FZD6), to enhance its pro-

tein stability. PCAT-1 activated Wnt/β-catenin signaling to facilitate AML progression in an FZD6-mediated manner. Our data provide a deeper molecular understanding of AML progression and implicate PCAT-1 as a potential novel therapeutic target.

Materials and methods

Ethical statement

In this study, all protocols were approved by the Ethical Review Board of the Affiliated Hospital of Qingdao University and the 971 Hospital of the Chinese People's Liberation Army. All experiments were carried out in accordance with the Declaration of Helsinki. And all participating patients signed consent forms.

Clinical specimens and cell culture

The use of human specimens in this study was sanctioned by the local ethics committee at the 971 Hospital of the Chinese People's Liberation Army. Bone marrow samples were collected from 58 AML patients and 30 healthy donors. According to the French-American-British (FAB) classification, 11 patients had AML M0, 11 had M1/M2, 15 had M3, 7 had M4/5 and 14 had M6/7. The sample was collected before the receiving of chemotherapy induction protocol. The human AML cell lines (Kasumi-6, HL-60, MOLT-3, AML-193 and BDCM) and human bone marrow stromal cells (HS-5) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) which contains 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) at 37°C in a humidified atmosphere with 5% CO_2.

Vectors and cell transfection

The sequence of short-hairpin RNA (shRNA) directed against PCAT-1 (sh-PCAT-1#, 5'-AUACAUAAGACCAUGGAAAU-3'; sh-PCAT-1##, 5'-GCTCACGCCTGTAATCTCA-3'; sh-PCAT-1###, 5'-AUACAUAAGACCAUGGAAAU-3') or negative control shRNA (sh-NC, 5'-CCGCAGGTATGCACGCGT-3') were ligated into the pLKO.1-Puro vector (Takara, Tokyo, Japan). To overexpression of FZD6, the synthesized and purified FZD6 gene fragment was inserted into the expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA). Cells were added into 96-well plates (5×10^3 cells per well) or 6-well plates (1×10^5 cells per well). These oligonucleotides were transfected into Kasumi-6 and HL-60 by using Lipofectamine RNAiMAX (Invitrogen) in accordance with the manufacturer's protocol. The transfection efficiency was validated by RT-qPCR analysis or western blot analysis.

Quantitative RT-PCR (RT-qPCR)

Total RNA samples were extracted from bone marrow samples or cultured cells using TRIzol reagent (Invitrogen). cDNA was prepared from 1 μg of total RNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Tokyo, Japan). The PCR amplifications were carried out using the SYBR Premix Ex Taq II (Takara) with Applied Biosystems 7500 Real-Time PCR System using the following gene-specific primers: PCAT-1 Forward, 5'-AATGGCATGAACCTGGGAGGCG-3', PCAT-1 Reverse, 5'-GGCTTTGGGAAGTGCTTTGGAG-3'; FZD6 Forward, 5'-ATGGAAAGGTCCCCGTTTCTG-3', FZD6 Reverse, 5'-GGGAAGAACGTCATGTTGTAAGT-3'; GAPDH Forward, 5'-AGGTCGGTGTGAACGGATTTG-3', GAPDH Reverse, 5'-TGTAGACCATGTAGTTGAGGTCA-3'. The $2^{-\Delta\Delta Ct}$ method [11] was used to measure the transcript levels of target genes and GAPDH acted as the internal control.

Cell proliferation assay

The proliferation of the AML cells in each group was detected using Cell Counting Kit-8 kit (CCK-8, MedChemExpress, Shanghai, China). After transfection, about 5000/ml Kasumi-6 and HL-60 cells were cultured into 96-well plates and allow to grow 0, 24, 48, 72 and 96 h. At the end of each point, cells were incubated with 10 μL CCK-8 solution in each well for an additional 2 h at 37°C. optical density (OD) at a wavelength of 450 nm was measured using a full wavelength microplate analyzer (BioTek, Richmond, USA).

Cell cycle analysis

After the cells were collected and fixed with 0.4% formaldehyde for 10 mins at room temperature, they were stained with propidium iodide (PI) solution for 30 min at 4°C and then analyzed using a FACSCalibur flow cytometer (Becton Dickinson and Company, CA, USA). ModFIT 3.0 software (Becton Dickinson) was employed for the calculation of cell cycle distribution.

Apoptosis analysis

To quantify the proportion of apoptotic cells in each group, AML cells were cultured on 6-well plates. After treatment, cells were harvested, washed with PBS, and then incubated with 5 μL Annexin V-FITC and 5 μL PI for 15 min in the dark at room temperature. After adding 400 μL binding buffer, the cells were analyzed by the flow cytometer within 1 h.

RNA pull-down

RNA pull-down assay was performed with the Pierce Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific) as described previously [12]. Biotin-labeled PCAT-1 truncation probe was incubated with streptavidin magnetic beads at 4°C overnight. The cell lysates were then interacted with biotinylated RNA for 4 h at 4°C. The level of FZD6 protein in the eluted complex was detected by western blot.

RIP (RNA immunoprecipitation) assay

RIP was performed using the EZ-Magna RIP kit (Millipore, USA) in accordance with the manufacturer's protocol. Cells were collected and lysed in complete RIPA buffer, followed by centrifugation and was incubated with antibody against FZD6 (Abcam), with anti-IgG (Abcam) as control. After incubation, cell lysate was then added with beads conjugated with anti-FZD6. Subsequently, co-precipitated RNAs were purified with RNeasy Mini Kit (QIAGEN, Duesseldorf, Germany) and detected by quantitative RT-PCR.

Promoter reporter and dual-luciferase assay

The FZD6 promoter was cloned into the pGL3 basic luciferase reporter vectors (Promega, Madison, WI, USA). For luciferase activity detection, about 5000 cells were cultured in 96-well plates and transfected with 100 ng TOP/FOP-flash reporter plasmids (Millipore, Billerica, MA, USA), 100 ng of an expression vector (pGL3-FZD6 or pGL3-Basic) or 0.25 μl siRNA. FZD6 promoter activity and TOP/FOP-flash were normalized by co-transfection with 10 ng Renilla luciferase reporter. After incubation for 24 h, the luciferase activity was detected using the Dual-Luciferase Reporter Assay System (Promega).

Western blot

The cells were lysed in RIPA Lysis and Extraction buffer (Thermo Fisher Scientific). Equal amounts of proteins were separated in by 12% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Thermo Fisher Scientific). The membranes were blocked with 5% skim milk at room temperature for 1 h and then incubated with primary antibodies against FZD6 (ab98933, 1:1000), β-catenin (ab6302, 1:1000), cyclin D1 (ab134175, 1:1000), c-myc (ab32072, 1:1000), and β-actin (ab8227, 1:1000, all obtained from Abcam) antibodies at 4°C overnight. Next day, the membranes were incubated with HRP-coupled goat anti-mouse or goat anti-rabbit secondary antibody (sc-2005, 1:2000, sc-2030, 1:5000; Santa Cruz, CA). ECL chemiluminescence kit (Thermo Fisher Scientific) was used to observe all proteins. Bio-Rad software was used to quantify each band.

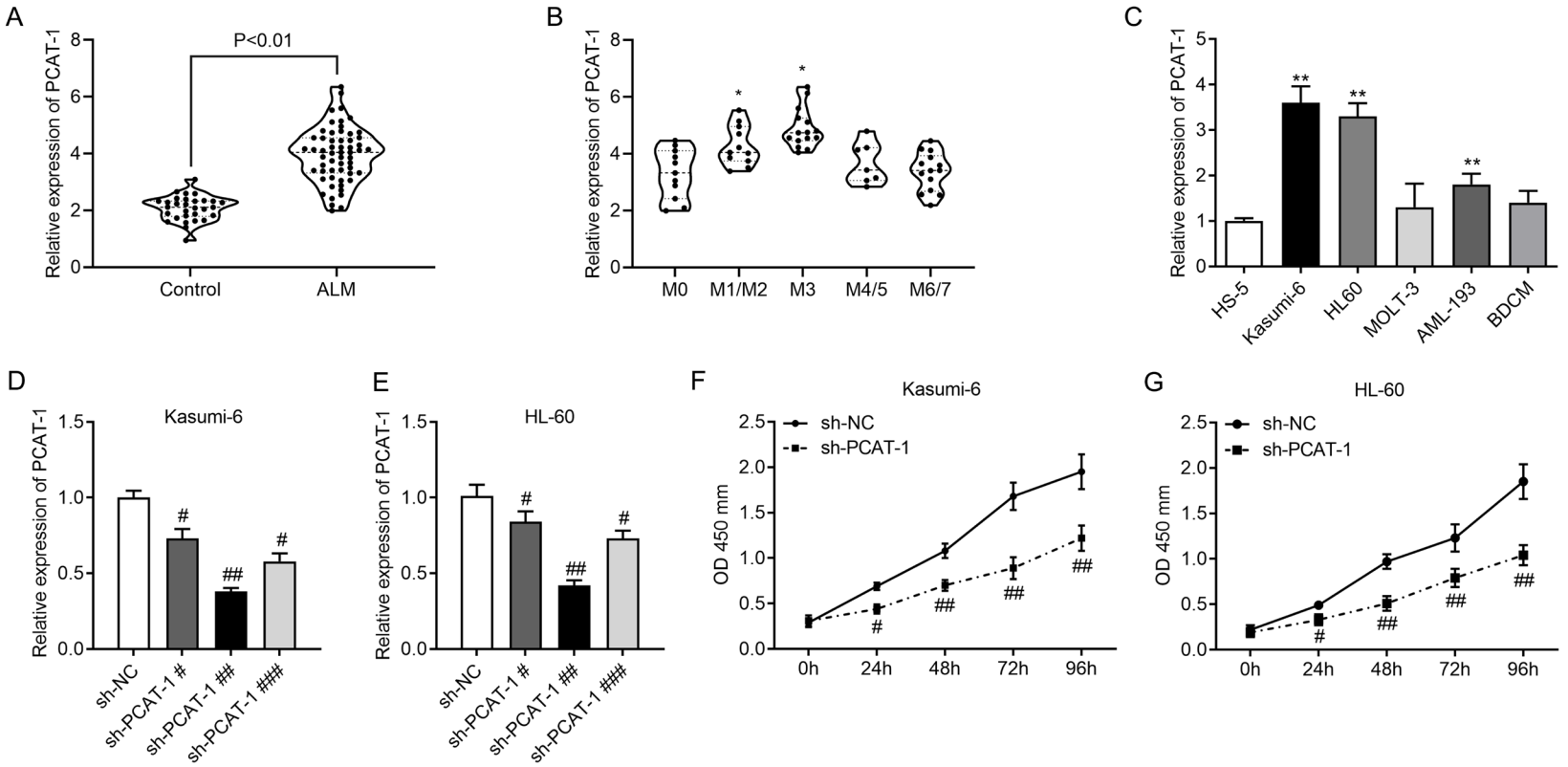
Statistical analysis

Data are presented as the mean ± standard deviation from at least three independent experiments. All experimental data were analyzed using SPSS 21.0 software (IBM Corp. Armonk, NY, USA). A two-tailed t-test was employed for the comparison between two groups. One-Way ANOVA was used for the comparison among multiple groups. The Pearson correlation was calculated between the expression levels of PCAT-1 and FZD6 in AML samples. *P* value <0.05 was considered to be statistically significant.

Results

Knockdown of PCAT-1 inhibits proliferation, induces the cycle arrest and cell apoptosis of AML cells

Firstly, RT-qPCR was performed to determine PCAT-1 level in AML specimens and in AML cell lines. The results revealed that compared with healthy controls, PCAT-1 was significantly increased in the bone marrow sample from AML patients (**Figure 1A**). The data in **Figure 1B** further demonstrated that PCAT-1 expression was differed in the FAB subtypes and especially increased in M1/2 and M3 type. Similarly, compared with bone marrow stromal cells (HS-5) cells, PCAT-1 was notably increased in M2 type (Kasumi-6) and M3 type (HL-60) cell lines, which were chosen for subsequent analysis (**Figure 1C**). To investigate the biofunctions of PCAT-1 in NSCLC, we knockdown of PCAT-1 using specific shRNA in Kasumi-6 and HL-60


A
P<0.01
Relative expression of PCAT-1
Control
ALM
B
Relative expression of PCAT-1
M0
M1/M2
M3
M4/5
M6/7
C
Relative expression of PCAT-1
HS-5
Kasumi-6
HL60
MOLT-3
AML-193
BDCM
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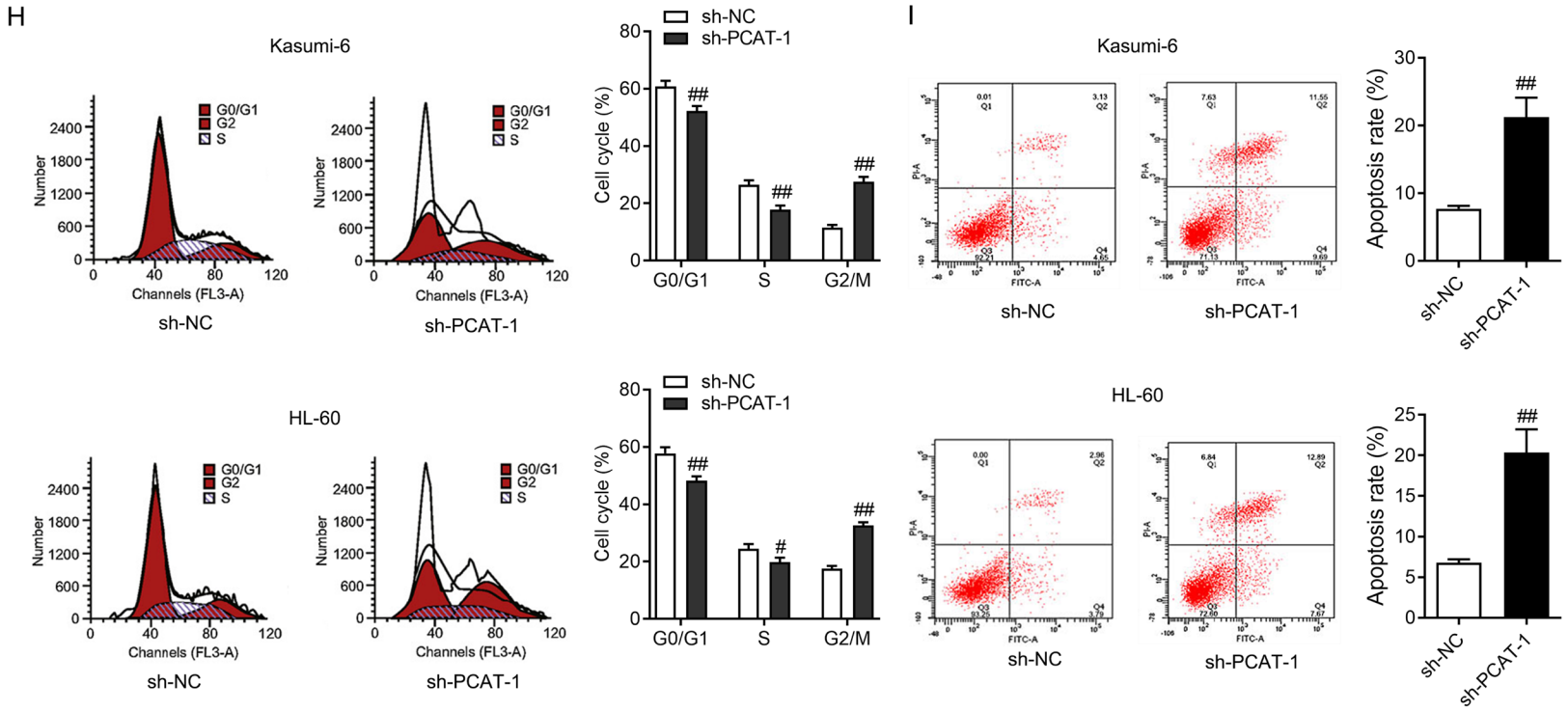


Figure 1. Knockdown of PCAT-1 suppressed the proliferation, induces the cycle arrest and accelerated the apoptosis of AML cells. A. Expression of PCAT-1 was analyzed by RT-qPCR in 58 AML patients (AML group) and 30 healthy donors (control group). B. PCAT-1 expression in the French-American-British (FAB) subtype of M1-M7. C. Expression of PCAT-1 was analyzed by RT-qPCR in five AML cell lines (Kasumi-6, HL-60, MOLT-3, AML-193 and BDCM) and human bone marrow stromal cells (HS-5). D, E. Expression of PCAT-1 was analyzed by RT-qPCR after introducing shRNA against PCAT-1 or the control shRNA (sh-NC) into Kasumi-6 and HL-60 cells. F, G. Cell proliferation of Kasumi-6 and HL-60 cells was detected through a CCK-8 kit after knockdown of PCAT-1. H. Cell cycles of the AML cells were detected through flow cytometry and the cell ratios of the G0/G1, S, G2/M phases in the Kasumi-6 and HL-60 cells after knockdown of PCAT-1 were indicated. I. Flow cytometry was used to detect cell apoptosis of AML cells. Q2 and Q4 square indicated the early and late apoptosis cells. *P<0.05 vs. M0; **P<0.01 vs. HS-5; #P<0.05, ##P<0.01 vs. sh-NC.

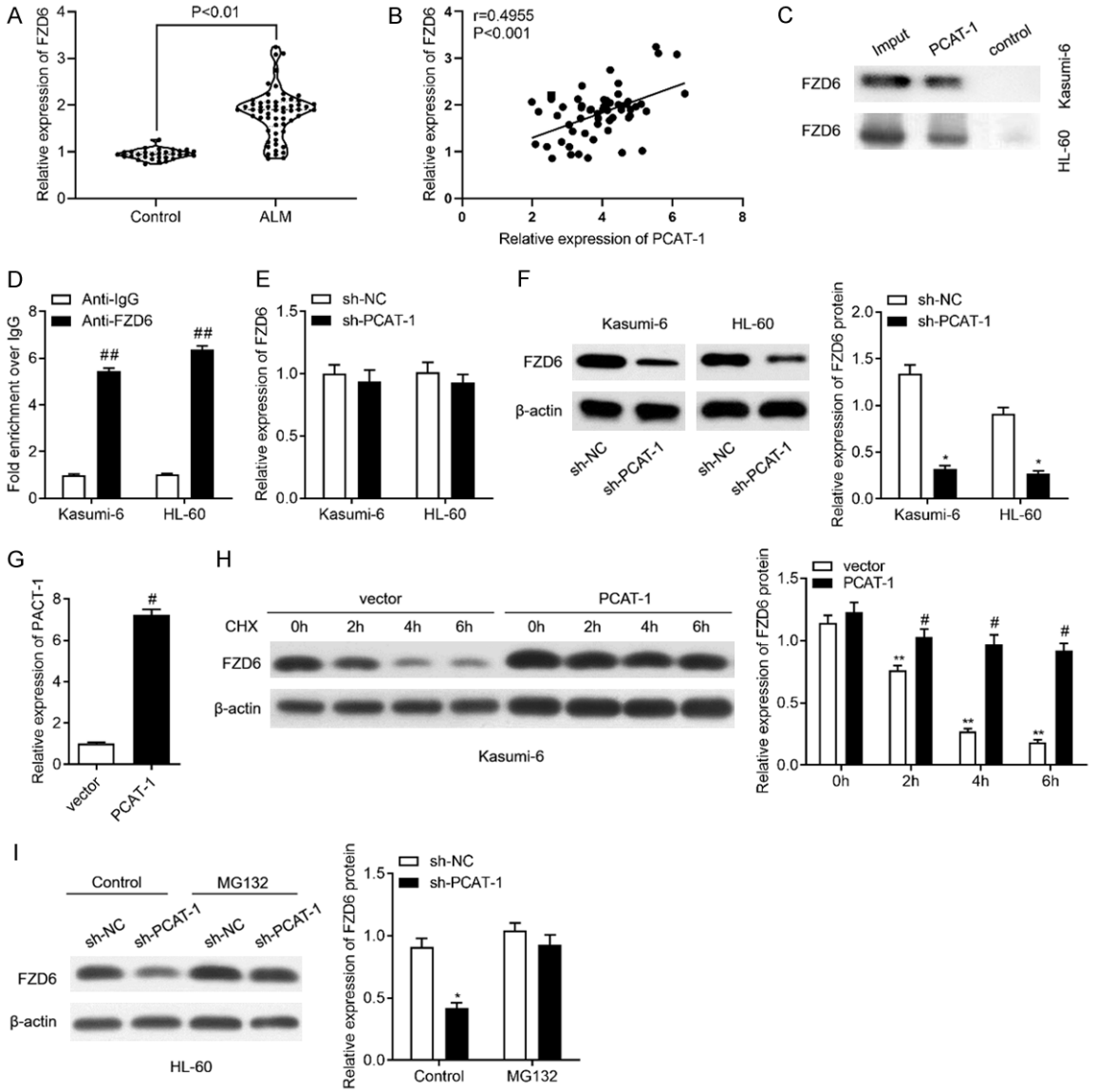


Figure 2. PCAT-1 interacted and enhanced with FZD6 stability. (A) Expression of FZD6 was analyzed by RT-qPCR in 58 AML patients (AML group) and 30 healthy donors (control group). (B) Correlation of PCAT-1 and FZD6 in 58 AML samples were analyzed using Pearson correlation analysis. r = 0.4955, P<0.01. (C) Kasumi-6 and HL-60 cells were transfected with PCAT-1 constructs for 24 h, and then the interactions between PCAT-1 constructs and FZD6 were detected by RNA pull-down assay or (D) RIP assay. (E) Expression of FZD6 in AML cells with or without PCAT-1 silencing, detected by RT-qPCR or (F) western blot assay. (G) Expression of PCAT-1 in Kasumi-6 cells with or without transfection with plasmid carrying PCAT-1 gene. (H) Expression of FZD6 protein after treatment of the Kasumi-6 cells with CHX (50 μg/mL). (I) Expression of FZD6 protein after treatment of the HL-60 cells with MG132 treatment (10 μmol/mL). *P<0.05 vs. sh-NC; **P<0.05 vs. 0 h; #P<0.05 vs. vector; ##P<0.05 vs. anti-IgG.

cells and the results showed that sh-PCAT-1## had the best inhibitory efficiency, which was used for the following experiments (**Figure 1D** and **1E**). Interestingly, we found that compared to shRNA negative control (sh-NC) treatment, knockdown of PCAT-1 significantly reduce the proliferation of AML cells (**Figure 1F** and **1G**). In addition, we found that knockdown of PCAT-1 caused an apparent G2/M arrest and the percentage of cells distributed in G0/G1 or S phases were decreased in both Kasumi-6 and HL-60 cells (**Figure 1H**). As displayed in **Figure 1I**, cell apoptotic rate in sh-PCAT-1 groups was notably increased when compared with the sh-NC group in AML cells. Taken together, these data suggested that knockdown of PCAT-1 inhibited

cell proliferation, arrested cell cycle progression and triggered apoptosis of AML cells.

PCAT-1 binds to the FZD6 protein and enhances its stability

In order to reveal the underlying mechanisms of the effects of PCAT-1 on AML cells, we used RPISeq online software (http://pridb.gdcb.iastate.edu/RPISeq/) to predict the interaction between PCAT-1 and proteins. Finally, we focused on FZD6, which is overexpressed in several cancers [13]. As shown in **Figure 2A**, FZD6 mRNA was significantly increased in AML specimens when comparable to the control. And further analysis revealed that PCAT-1 expression was positively collated with FZD6 expression (**Figure 2B**). Subsequently, RNA-protein pull-down assay confirmed that FZD6 directly bound to PCAT-1 in AML cells (**Figure 2C**). And the RIP assay confirmed the interaction between FZD6 and PCAT-1 in both Kasumi-6 and HL-60 cells (**Figure 2D**). The regulatory effects of PCAT-1 on FZD6 were then evaluated. The results showed that knockdown of PCAT-1 could reduce the FZD6 protein level but not the mRNA level in AML cells (**Figure 2E** and **2F**), indicating that PCAT-1 might regulate FZD6 at the posttranscriptional level. Furtherly, we used the protein synthesis inhibitor cycloheximide (CHX) to observe the effect of PCAT-1 on FZD6 degradation. Upregulation of FZD6 in Kasumi-6 cells was confirmed by RT-qPCR assay (**Figure 2G**) and the results in **Figure 2H** showed that PCAT-1 overexpression enhanced FZD6 protein stability. Furthermore, the 26S proteasome inhibitor MG132 rescued the reduction of FZD6 caused by repression of PCAT-1 in HL-60 cells (**Figure 2I**), suggesting that PCAT-1 elevated FZD6 by reducing its degradation. Above data showed that PCAT-1 directly bound the FZD6 protein and enhanced its stability in AML cells.

PCAT-1 regulates AML cell function in an FZD6-mediated manner

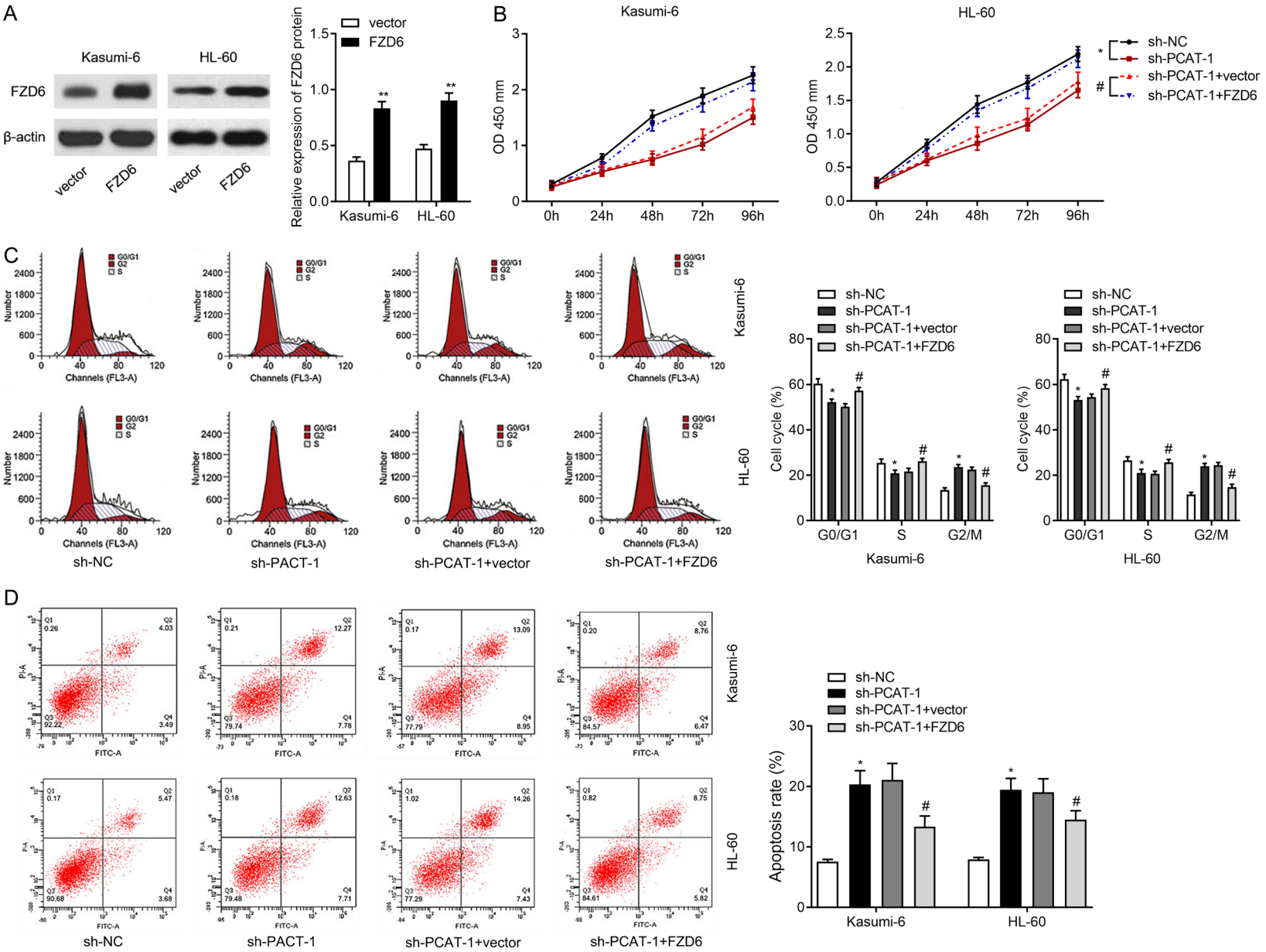
We subsequently analyzed whether the role of PCAT-1 on AML cells was dependent on FZD6. The results in **Figure 3A** depicted that overexpression of FZD6 increased FZD6 protein expression in AML cells. Moreover, FZD6 overexpression reversed the inhibition of PCAT-1 on cell proliferative rate in Kasumi-6 and HL-60 cells (**Figure 3B**). Meanwhile, flow cytometry revealed that transfection of FZD6 partially reversed the effect of PCAT-1 on cell cycle progression of AML cells (**Figure 3C**). Moreover, the increased percentage of apoptotic cells due to PCAT-1 knockdown was offset by FZD6 overexpression (**Figure 3D**). Therefore, we believed that PCAT-1 exerted its function on AML cells in an FZD6-mediated manner.

The influence of PCAT-1 on β-catenin activation was dependent on FZD6

A recent study reported that PCAT-1 silencing could inhibit Wnt/β-catenin signaling pathway activity in extrahepatic cholangiocarcinoma [14]. In the present study, we performed a TOP/FOP-flash luciferase assay and found that Wnt/β-catenin signaling was inhibited by knockdown of PCAT-1 (**Figure 4A**). The results of RNA pull-down in the **Figure 4B** further identified an interaction between FZD6 and β-catenin in HL-60 cells. Moreover, to confirm the regulatory effects of FZD6 on Wnt/β-catenin signaling, the levels of c-myc and cyclin D1 were detected by western blot. The results found that knockdown of PCAT-1 reduced the protein levels of Wnt/β-catenin signaling target proteins (c-myc and cyclin D1) and overexpression of FZD6 partly reversed the inhibitory effects of PCAT-1 depletion on Wnt/β-catenin signaling (**Figure 4C-E**). Collectively, these results suggested that PCAT-1 indirectly promotes β-catenin transcriptional activation by binding to FZD6 in AML cells.

Discussion

lncRNA PCAT-1 has been identified to be an oncogenic RNA in multiple human cancers. PCAT-1 was found upregulated in ovarian cancer and PCAT-1 promoted proliferation and inhibited apoptosis of ovarian cancer cells [15]. Additionally, PCAT-1 overexpression promoted proliferation, migration, invasion and inhibited apoptosis of prostate cancer cells [16]. Nevertheless, the expression and functions of PCAT-1 in AML have not been clarified yet. In this study, we firstly examined the expression profile of PCAT-1 and its role in AML and revealed that PCAT-1 was upregulated in AML specimen and cell lines. Knockdown of PCAT-1 inhibited proliferation, induced the cycle arrest and cell apoptosis of AML cells. Thus, we further investigated the underlying mechanisms of the role of PCAT-1 on AML cells.


A
Kasumi-6
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sh-NC
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sh-PCAT-1+vector
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C
G0/G1
G2
S
Number
Channels (FL3-A)
Kasumi-6
HL-60
sh-NC
sh-PACT-1
sh-PCAT-1+vector
sh-PCAT-1+FZD6
Cell cycle (%)
G0/G1
S
G2/M
D
PI-A
FITC-A
Apoptosis rate (%)

Figure 3. PCAT-1 exerted its role in AML cells in a DDX5-mediated manner. A. Expression of PCAT-1 was analyzed by western blot after overexpression of FZD6 in Kasumi-6 and HL-60 cells. B. Cell proliferation of Kasumi-6 and HL-60 cells was detected through a CCK-8 kit after knockdown of PCAT-1 and/or FZD6 overexpression. C. Cell cycles of the AML cells were detected through flow cytometry and the cell ratios of the G0/G1, S, G2/M phases in the Kasumi-6 and HL-60 cells after knockdown of PCAT-1 and/or FZD6 overexpression were indicated. D. Flow cytometry was used to detect cell apoptosis of AML cells. Q2 and Q4 square indicated the early and late apoptosis cells. *P<0.05 vs. sh-NC; **P<0.05 vs. vector; #P<0.05 vs. sh-PCAT-1.

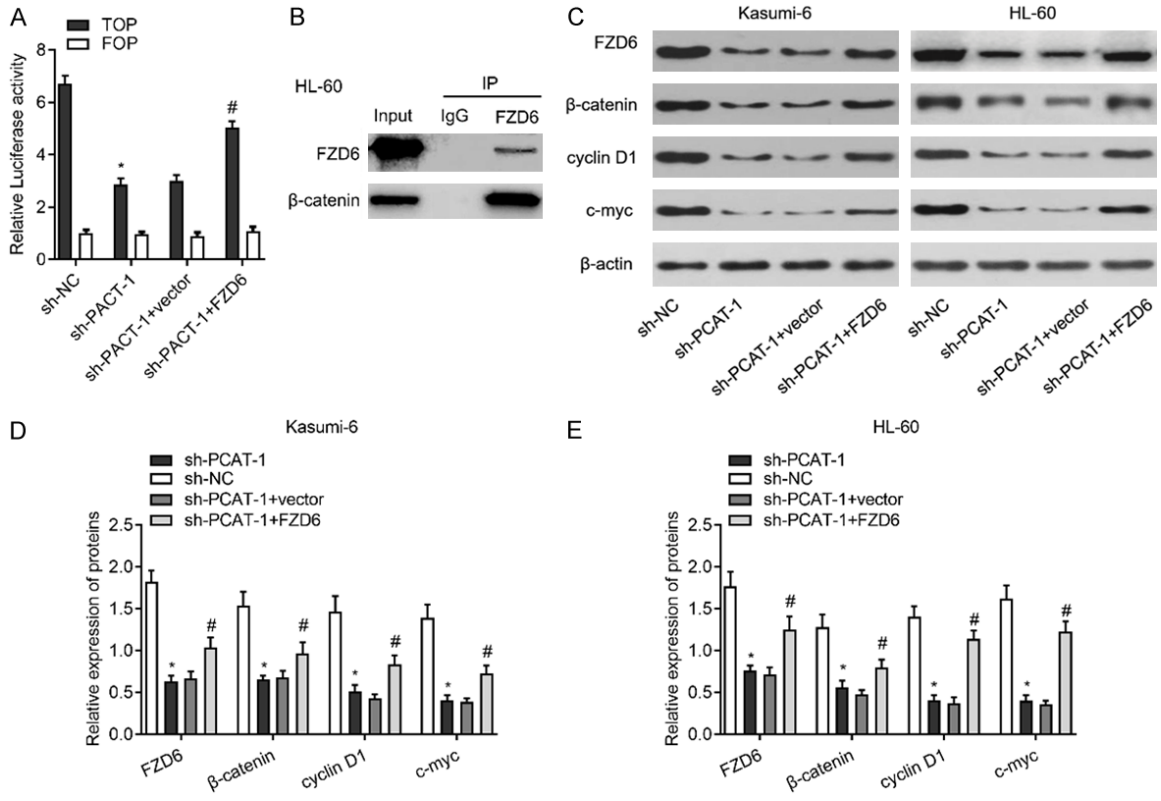

Figure 4. PCAT-1 activated Wnt/β-catenin signaling pathway through interacting with DDX5. A. HL-60 cells were transfected with TOP-Flash or control FOP-Flash reporter to determine reporter activities 48 h later after knockdown of PCAT-1 and/or FZD6 overexpression. B. Co-IP assay was used to detect the interaction of FZD6 and β-catenin in HL-60 cells. C-E. Western blot analysis showed the FZD6, β-catenin, cyclin D1, and c-myc protein levels in Kasumi-6 and HL-60 cells after knockdown of PCAT-1 and/or FZD6 overexpression. *P<0.05 vs. sh-NC; #P<0.05 vs. sh-PCAT-1.

It is well established that LncRNAs exert their functions mainly by binding to their corresponding proteins [17]. Using RNA-protein interaction prediction software RPISeq (http://pridb.gdcb.iastate.edu/RPISeq/) [18], we assumed that PCAT-1 may interact with frizzled class receptor 6 (FZD6) in AML cells. FZD6 belongs to the frizzled family and, in general, frizzled family proteins expose their large N-terminus on the extracellular side containing a cysteine-rich domain that binds the receptor's ligands [19]. FZD family of proteins is the most important receptor family of the Wnt/β-catenin pathway, and abnormal expression of FZDs was closely related to carcinogenesis [20]. Aberrant activation of the Wnt/β-catenin pathway and its downstream targets have been reported to play a role in AML progression [21, 22]. Therefore, we investigated whether PCAT-1 affected β-catenin in an FZD6-dependent manner.

It was reported that PCAT-1 could negatively regulate the BRCA2 tumor suppressor protein and positively regulate c-myc oncoprotein [23]. Moreover, decreased PCAT-1 expression was found to inhibit Wnt/β-catenin signaling pathway activity [14]. However, PCAT-1 transcripts are mostly located in the cytoplasm [24], suggesting that it may active Wnt/β-catenin pathway in an indirect way. A recent study reported

that neuronal pentraxin 2 was shown to interact with FZD6 to promote β-catenin translocation into the cell nucleus, resulting in an increase in the expression of c-myc, cyclin D1 and N-cadherin [25]. Using TOP/FOP-flash luciferase assay and RNA pull-down assay, we confirmed that lncRNA PCAT-1 could directly interact with FZD6 to enhance its stability in the present study. We validated that the PCAT-1 active Wnt/β-catenin signaling pathway in an FZD6-dependent manner. Moreover, we further demonstrated that overexpression of FZD6 in AML cells resulted in a rescue action on PCAT-1 silencing induced inhibition of Wnt/β-catenin signaling pathway.

There are limitations to this study. Firstly, because the Wnt/β-catenin pathway is a very complex system, we only demonstrated the interaction between PCAT-1 and FZD6, but did not identify the effects of PCAT-1 on other receptors or ligands, such as Wnt ligand family. Secondly, further studies are needed to confirm that upregulation of PCAT-1 could directly active Wnt/β-catenin signaling pathway though interacting with FZD6.

Taken together, this study reveals that knockdown of PCAT-1 inactivated Wnt/β-catenin transcriptional activity by directly interacting with FZD6, which might subsequently inhibit cell proliferation, arrested cell cycle progression and triggered apoptosis of AML cells. Our findings elucidate a potential molecular basis of PCAT-1 in AML progression and providing a promising therapeutic target for AML.

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Disclosure of conflict of interest

None.

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