TRIB2 knockdown as a regulator of chemotherapy resistance and proliferation via the ERK/STAT3 signaling pathway in human chronic myelogenous leukemia K562/ADM cells

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Received September 26, 2017; Accepted January 31, 2018

DOI: 10.3892/or.2018.6249

Abstract. Acquired resistance to chemotherapy plays a critical role in human drug treatment failure in many tumor types. Multidrug resistance (MDR) to Adriamycin (ADM) also limits the efficacy of therapy in human chronic myelogenous leukemia (CML). The overexpression of drug efflux transporters is one mechanism underlying MDR. In particular, the consistent activation of MDR1 and MDR-associated protein 1 (MRP1) is involved in drug resistance. In the present study, ADM-resistant human CML K562/ADM cells were stably transfected with a Tribbles homolog 2 (TRIB2)-targeted vector. A CCK-8 assay showed that the half maximal inhibitory concentration (IC50) of ADM and the cell proliferation were lower in the transfected cells compared with that in the parental K562/ADM cells. The mRNA and protein expression levels of MDR1 and MRP1 were determined by reverse transcription-polymerase chain reaction (RT-PCR), RT-quantitative PCR and western blot analysis. The results showed that the expression of MDR1 and MRP1 was significantly reduced in K562/ADM cells transfected with pGPU6/GFP/Neo-TRIB2. Due to the downregulation of MDR1 and MRP1, the intracellular accumulation of ADM was increased in the transfected cells compared with that in the parental K562/ADM cells. Therefore, the sensitivity of the K562/ADM cells to ADM was enhanced and proliferation was inhibited. Our research revealed that protein expression of the ERK signaling pathway was inhibited by downregulating TRIB2, indicating that the ERK pathway was involved in cell drug resistance and proliferation. Furthermore, we used the ERK-specific blocker U0126 to demonstrate this phenomenon. In summary, our research suggested that knockdown of TRIB2 could slow cell growth and reverse resistance, implying that TRIB2 is a potential therapy target for resistant human CML.

Introduction

Chronic myeloid leukemia (CML) is one of the most prevalent types of myeloproliferative neoplasm, and its multidrug resistance (MDR) is usually associated with a poor clinical outcome (1,2). MDR1 and MRP1 belong to the protein family of ATP-binding cassette transporters, which use the energy released by ATP hydrolysis to bind drugs and export them from the cell (3,4). Adriamycin-resistant CML K562 (K562/ADM) cells reportedly overexpress MDR1 and MRP1 (5), meaning that the resistance of this cell line is associated with abnormalities in drug efflux. In the present study, we found that the proliferation of K562/ADM cells was significantly inhibited upon knockdown of the Tribbles homolog 2 (TRIB2) gene, compared with that noted in the untreated cells. The reason for this growth inhibition in the resistant cell line may be associated with cell drug-resistance reversal. It is known that TRIB2 is expressed in mammals. TRIB protein family members encode pseudo-kinase proteins that are highly conserved in evolution, and act as adaptors in signaling pathways for important cellular processes (6,7). Previous studies have focused on the pathological role of TRIB2 in various diseases, including CML, and metabolic and neurological diseases, in which it has been identified as a critical signaling modulator and mediator (8,9). Related reports
suggest that TRIB2 overexpression can indeed promote tumor resistance by activating relevant cell pathways (10). However, little is known concerning the effects of TRIB2 gene knockdown on drug-resistant proteins.

Previous studies have found that the downregulation of drug-resistance proteins may partly depend on inhibition of the ERK pathway in cancer (11-13). In the present study, we explored changes in the ERK signaling pathway after knockdown of the TRIB2 gene in K562/ADM cells. The aim of the present study was to explore the effect of the downregulation of TRIB2 expression on the chemotherapy resistance and proliferation of K562/ADM cells. The results provide a novel basis for the treatment of clinical drug resistance mechanisms, and potential routes for therapeutic strategies in CML.

Materials and methods

Cell lines and cell culture. Human CML K562 cells and the MDR sub-cell line K562/ADM cells were obtained from the Institute of Medical Molecular Genetics of Binzhou Medical University (Yantai, China). The cells were maintained in RPMI-1640 basic medium (1X) supplemented with 10% fetal bovine serum (FBS; both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere containing 5% CO₂.

K562/ADM cells were maintained in the presence of 7 µM ADM (Wanle, Shenzhen, China). Prior to the experiment, the cells were cultured in drug-free medium for 1 week. In addition, K562/ADM and K562/ADM-TRIB2 cells were pretreated for 24 h with 10 µM U0126 (Shanghai Selleck Chemicals Co., Ltd., Shanghai, China) in order to study the ERK pathway.

Cell transfection. A pair of vector sequences targeting TRIB2 were generated and named pGPU6/GFP/Neo-TRIB2 and pGPU6/GFP/Neo-shNC-1. Firstly, cells were seeded at a density of 0.9-4x10^5/well into incubation. After incubating for a further 6-18 h, the medium K562/ADM cells (8x10^3) with or without knockdown of TRIB2 were divided into 0, 24, 48 and 72 h experimental groups, seeded into 96-well plates and incubated with medium containing 10% FBS. Each group consisted of five parallel wells, with parental K562/ADM cells serving as the control. Then, 10 µl CCK-8 (Dojindo Molecular Technologies, Inc., Shanghai, China) solution was added to each well and incubated for a further 4 h. Then, the absorbance was measured at 450 nm using a fluorescence spectrophotometer (F-7000; Hitachi, Ltd., Tokyo, Japan). The absorbance values were collected for processing using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA).

CCK-8 analysis of the half maximal inhibitory concentration (IC₅₀). The CCK-8 was used to determine the inhibition ratio of cells incubated with various concentrations of ADM (0-16 µM for the K562 cells; 15-120 µM for the K562/ADM cells; 15-72 µM for the K562/ADM-Con cells or K562/ADM-TRIB2 cells). After incubation in RPMI-1640 medium for 24 h, 10 µl CCK-8 solution was added to each well and incubated for 4 h. The absorbance was then measured at 450 nm with a microplate reader. A blank well containing only medium and ADM were used as a control. The concentration of ADM that resulted in the IC₅₀ was calculated. Resistant fold=IC₅₀ K562/ADM/IC₅₀ K562. Reversal fold=IC₅₀ K562/ADM-TRIB2 group/IC₅₀ K562 control.

Flow cytometry. K562, K562/ADM, and cells transfected with pGPU6/GFP/Neo-TRIB2 and pGPU6/GFP/Neo-shNC were seeded into a 6-well plate at a density of 5x10^5 cells/well. The non-transfected group was defined as the blank control. A total of 5 µM ADM was applied to the wells. After incubation for 1 h, the cells were harvested via centrifugation and washed twice with ice-cold PBS. The cell-associated mean fluorescence intensity (MFI) of ADM was detected using a FACScalibur flow cytometer (FACS FC500; Beckman Coulter, Inc., Brea, CA, USA), with excitation/emission wavelengths of 485/580 nm.

Reverse transcription-polymerase chain reaction (RT-PCR) and RT-quantitative PCR (RT-qPCR). According to the manufacturer's instructions, total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Inc.) and assessed at a ratio of A260/A280 by spectrophotometry (Nano Drop 2000; Nano Drop Technologies, Inc., Wilmington, DE, USA). RNA (1-2 µg) was used to synthesize the first-strand cDNA. The primers used in this experiment were designed and synthesized by Shanghai GenePharma, Co., Ltd. (Shanghai, China) and are presented in Table I. Prime Script™ RT reagent kit with gDNA Eraser (Takara Bio, Inc., Otsu, Japan) was used to perform the RT reaction. Then, Premix Taq™ (Takara Bio, Inc.) was used to perform PCR amplification on the Eppendorf Mastercycler Personal system (Eppendorf China Ltd., Hong Kong, China). The reaction system contained forward primer, reverse primer, Premix Taq and template cDNA. The PCR products were separated on 1% agarose gels (Takara Bio, Inc.), stained with G-Red nucleic acid dyes (1:10,000; BioTeke Corporation, Beijing, China). The images were captured with a Tanon gel imaging system (Tanon, Shanghai, China). The parental non-transfected K562/ADM cells were used as the blank control. GAPDH served as an internal standard for quality control and quantification of target genes.

RT-qPCR was performed with a StepOne™ Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and SYBR Premix Ex Taq™ (Takara Bio, Inc.). The reaction system of PCR contained SYBR Premix Ex Taq™, the forward primer, the reverse primer, template cDNA and nuclease-free distilled water. The results were calculated using the 2^ΔΔCq value.

Western blot analysis. Cells cultured in 6-well plates were harvested and washed with cold PBS three times. A total of
120 µl RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) was added to extract the proteins. Protein samples were separated via 10 or 6% SDS-PAGE (Beyotime Institute of Biotechnology) with a constant voltage of 80 V for 0.5 h, which was then switched to 120 V for a further 1 h. Proteins were then transferred onto polyvinylidine difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed dry milk in 1X Tris-buffered saline with Tween-20 (pH 8.0) at room temperature for 2 h, and then incubated overnight at 4˚C with six styles of primary antibodies respectively. The primary antibodies were rabbit polyclonal anti-TRIB2 (1:500; cat. no. 204119; Abcam, Cambridge, UK), anti-MDR1 (1:500; cat no. 0563R; BIOSS, Beijing, China), anti-MRP1 (1:500; cat. no. 0657R; BIOSS), anti-STAT3 (1:500; cat. no. 1141R; Bioworld Technology, Inc., Nanjing, China), anti-p-ERK (1:500, cat no. 5016; Bioworld Technology, Inc.) and anti-GAPDH (1:1,000; cat. no. AP0063; Bioworld Technology, Inc.). Following this, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:5,000; cat no. 13278; Bioworld Technology, Inc.) for 2 h. Finally, images were captured using a FluorChem FC2 gel imaging system (Protein Simple, San Jose, CA, USA). The intensity of each band was normalized to GAPDH in the respective lane, and the K562/ADM cells were as control.

**Table I. Primers used in reverse transcription-quantitative polymerase chain reaction.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR1</td>
<td>Forward: 5’-GGAGCCTACTTGGTGCCACATAA-3’</td>
<td>121</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-TGGCATAGTGCAGGCAAATGAAC-3’</td>
<td></td>
</tr>
<tr>
<td>MRP1</td>
<td>Forward: 5’-CAGCCCCCTTCCTGACAAAGCTA-3’</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-GTGGGCTTCATCCAACACAGA-3’</td>
<td></td>
</tr>
<tr>
<td>TRIB2</td>
<td>Forward: 5’-CTCCGAACCTTGTCGATTGAA-3’</td>
<td>233</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CACATAGCCTTGGTCTCA-3’</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5’-CAGCCCCCTTCCTGACAAACAA-3’</td>
<td>133</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-GTGGGCTTCATCCAACACAGA-3’</td>
<td></td>
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</tbody>
</table>

MDR1, multidrug resistance 1; MRP1, MDR-associated protein 1; TRIB2, Tribble homologue 2.

**Table II. IC_{50} values for K562/ADM cells and K562 cells toward ADM by CCK-8 assay (means ± SD; n=5).**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC_{50} ± SD (µM)</th>
<th>K562</th>
<th>K562/ADM</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adriamycin</td>
<td>3.219± 0.921</td>
<td>84.801±0.0183a</td>
<td>26.22</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.01 vs. K562 group. RF, Resistant fold; ADM, Adriamycin.

120 µl RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) was added to extract the proteins. Protein samples were separated via 10 or 6% SDS-PAGE (Beyotime Institute of Biotechnology) with a constant voltage of 80 V for 0.5 h, which was then switched to 120 V for a further 1 h. Proteins were then transferred onto polyvinylidine difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed dry milk in 1X Tris-buffered saline with Tween-20 (pH 8.0) at room temperature for 2 h, and then incubated overnight at 4˚C with six styles of primary antibodies respectively. The primary antibodies were rabbit polyclonal anti-TRIB2 (1:500; cat. no. 204119; Abcam, Cambridge, UK), anti-MDR1 (1:500; cat no. 0563R; BIOSS, Beijing, China), anti-MRP1 (1:500; cat. no. 0657R; BIOSS), anti-STAT3 (1:500; cat. no. 1141R; Bioworld Technology, Inc., Nanjing, China), anti-p-ERK (1:500, cat no. 5016; Bioworld Technology, Inc.) and anti-GAPDH (1:1,000; cat. no. AP0063; Bioworld Technology, Inc.). Following this, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:5,000; cat no. 13278; Bioworld Technology, Inc.) for 2 h. Finally, images were captured using a FluorChem FC2 gel imaging system (Protein Simple, San Jose, CA, USA). The intensity of each band was normalized to GAPDH in the respective lane, and the K562/ADM cells were as control.

**Table III. Construction of the expression vector and its interference sequence (5’-3’).**

<table>
<thead>
<tr>
<th>Vector construction</th>
<th>Interference sequence</th>
</tr>
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<tbody>
<tr>
<td>pGPU6/GFP/Neo-shNC</td>
<td>GTTCTCCGAACGTGTCACGT</td>
</tr>
<tr>
<td>pGPU6/GFP/Neo-TRIB2</td>
<td>TACGGAGATATGGGAGATC</td>
</tr>
<tr>
<td>pGPU6/GFP/Neo-TRIB2-1</td>
<td>CTTGTCGACATCCGTTTCTTG</td>
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</table>

**Results**

Calculation of the level of ADM drug-resistance in the K562 and K562/ADM cell groups. The IC_{50} was calculated by performing a CCK-8 spectrophotometric assay. The data from the K562/ADM cells were markedly higher compared with K562 cells, with a resistance ratio of 26.22 for K562/ADM to K562 cells (Table II). There was a significant difference in intracellular ADM accumulation between the K562/ADM group and the K562 group (Fig. 1). All values were statistically significant (P<0.01).

Determination of relative protein expression in K562 and K562/ADM cells. We evaluated MDR1, MRP1 and TRIB2 expression in the non-resistant K562 cells and the ADM-resistant K562/ADM cells. The results indicated that the K562 and K562/ADM cells both showed high expression levels of all three proteins. The K562/ADM cells exhibited higher mRNA expression of TRIB2, MDR1 and MRP1, compared with the K562 cells (Fig. 2A and B). Western blot analyses revealed the same expression trends at the protein level (Fig. 2C and D).

Data analysis. Statistical analyses were performed using SPSS 21.0 software (IBM Corp., Armonk, NY, USA). Independent two-sample t-tests were used to analyze differences between two groups. One-way analysis of variance (ANOVA) was used to analyze differences among three or more groups.

Construction of a stable TRIB2 transfection cell system. To evaluate the functional changes in the K562/ADM cells following silencing of TRIB2, we transfected pGPU6/GFP/Neo-shNC, pGPU6/GFP/Neo-TRIB2 and pGPU6/GFP/Neo-TRIB2-1 (Table III) into K562/ADM cells, with untreated K562/ADM
cells serving as the control. Green fluorescence is only detected in cells successfully transfected with GFP. Therefore, cells with green fluorescence indicate a high efficiency of transfection. After 4 weeks of G418 selection, we successfully obtained stable positive clones (Fig. 3A). RT-PCR, RT-qPCR and western blot analyses revealed that TRIB2 expression was markedly decreased in the K562/ADM-TRIB2 group and the K562/ADM-TRIB2-1 group at the transcription and translation levels, compared with that in the K562/ADM group. TRIB2 expression in the K562/ADM-Con group was not significantly different from the negative control group (Fig. 4).

**TRIB2 knockdown inhibits cell proliferation.** A CCK-8 assay was performed to evaluate cell proliferation (Fig. 3B). According to the growth curve, we found that the proliferation of cells treated with pGPU6/GFP/Neo-TRIB2 and pGPU6/GFP/Neo-TRIB2-1 was markedly inhibited, while cells transfected with pGPU6/GFP/Neo-shNC exhibited no significant difference. The results also showed that pGPU6/GFP/Neo-TRIB2 was more effective than pGPU6/GFP/Neo-TRIB2-1. Then, pGPU6/GFP/Neo-TRIB2 was used to explore the effect of the downregulation of TRIB2 expression on the chemotherapy resistance and proliferation of K562/ADM cells.

**TRIB2 knockdown decreases IC_{50} and increases intracellular ADM accumulation.** The IC_{50} value was calculated by performing a CCK-8 spectrophotometric assay. The IC_{50} of the K562/ADM-Con group was not observed to be significantly different compared with the K562/ADM cells, while a reduction in the IC_{50} value was obvious in the K562/ADM-TRIB2 group, with a reversal fold of 12.12 (Table IV). The intracellular ADM accumulation in K562/ADM-TRIB2 cells was considerably higher than that in the K562/ADM cells and K562/ADM-Con cells (Fig. 5). All values were statistically significant (P<0.01).

**Decreased expression of MDR1 and MRP1 by TRIB2 knockdown.** MDR1 and MRP1 are ABC transporters overexpressed in many drug-resistant tumor cells, which contribute...
to the development of MDR. Therefore, we assessed whether TRIB2 knockdown could influence the expression of MDR1 and MRP1. Notably, western blotting, RT-PCR and RT-qPCR analyses (Fig. 6) illustrated that the expression levels of MDR1 and MRP1 were lower in the K562/ADM-TRIB2 cells, compared with levels in the control cells. This indicated that TRIB2 may be involved in key steps of MDR development in CML.

Inhibition of the ERK pathway in K562/ADM-TRIB2 cells. The expression of p-ERK and STAT3 in K562/ADM cells was higher compared with that noted in the K562 cells. However,
Figure 5. Analysis of intracellular Adriamycin accumulation in a stably transfected TRIB2 cell system. (A-C) No significant differences in intracellular accumulation of doxorubicin (Adriamycin) was observed between the K562/ADM-Con cells and the control group. However, the accumulation in K562/ADM-TRIB2 cells was markedly decreased. (D) ADM MFI demonstrated the differences between groups. All data are presented as the mean ± standard deviation (n=3). **P<0.01.

Figure 6. Analysis of MDR1 and MRP1 expression by RT-qPCR and western blotting. (A) mRNA expression of MDR1 and MRP1 was reduced significantly following TRIB2 knockdown. (B) Statistical analysis of RT-PCR optical density values. (C) Statistical analysis of RT-qPCR values. (D) MDR1 and MRP1 protein expression was lower in K562/ADM-TRIB2 cells compared with K562/ADM cells. (E) Optical density of MDR1 expression. (F) Optical density of MRP1 expression. GAPDH was used as an internal reference. Data are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 and ***P<0.001. MDR1, multidrug resistance 1; MRP1, multidrug resistance-associated protein 1; TRIB2, Tribble homologue 2.
after treatment with U0126, p-ERK and STAT3 expression was significantly decreased (Fig. 7A–C). These results suggested that expression of the ERK pathway was active in CML K562/ADM cells and U0126 could specifically block this pathway. Furthermore, the expression of p-ERK and STAT3 was clearly downregulated in the K562/ADM-TRIB2 cells. After treatment with U0126, the expression of p-ERK and STAT3 in K562/ADM-TRIB2 cells was significantly decreased, indicating that TRIB2 knockdown may act by blocking ERK pathway activity (Fig. 7D-F). These results suggest that downregulation of TRIB2 affects cell resistance by altering the expression of p-ERK and STAT3 in CML K562/ADM cells.

**Discussion**

MDR is a major clinical obstacle for effective tumor chemotherapy, and its impact on chemotherapy in the clinic is worsening. Therefore, novel targeted therapeutic approaches are being explored in order to increase the efficacy of chemotherapy against blood cancers and diseases (14). We observed that TRIB2, MDR1 and MRP1 expression levels were higher in K562/ADM cells, compared with levels in the K562 cells, at both the protein and mRNA levels. This indicated that TRIB2 was involved in the development of MDR in K562/ADM cells.

Numerous studies have shown that the HOX gene family plays an important role in tumor resistance. Knockdown of HOXA5, HOXA10 or HOXB4 was found to reverse multidrug resistance of human CML K562/ADM cells (2,15-17). Related studies have also shown that miR-3142 and miR-146a are overexpressed in K562/ADM cells compared with that noted in K562 cells, which promotes normal cell proliferation and enhanced resistance to ADM in vitro (18,19). Similarly, knockdown of miR-224 and let-7i was shown to reverse the MDR of human CML K562/ADM cells (20). It has been reported that TRIB2 expression is significantly increased in tumor tissues from patients, correlating with the increased phosphorylation of AKT, FOXO3a, MDM2 and C/EBPα (10,21). We found that the knockdown of TRIB2 could decrease MDR1 and MRP1 activity in human CML K562/ADM cells, and also reverse intracellular drug accumulation. In the present study, we first focused on evaluating the association between TRIB2 knockdown and the expression of resistance proteins. Our results indicated that the expression levels of drug-resistant proteins were inhibited by suppressing TRIB2, which reduced the efflux of intracellular ADM and reversed cell resistance. In summary, TRIB2 repression could partially reverse the MDR of K562/ADM cells by inhibiting cellular efflux functions and downregulating the expression levels of MDR1 and MRP1, thus elevating intracellular chemotherapeutic accumulation.

To further investigate the mechanism underlying the role of TRIB2 knockdown in reducing cell resistance, we examined the activity of the ERK pathway. ERK1 and ERK2 constitute the ERK1/2 signal transduction pathway. This is mainly composed of the RAS/RAF/MEK/ERK signal transduction cascade, which can be stimulated by various external stimuli (22,23). Expression of the ERK signal transduction
pathway and drug efflux proteins in hepatocellular carcinoma, gastric cancer and breast cancer cells is reported to be significantly higher compared with the corresponding controls (24-26). Numerous studies have shown that excessive activation of ERK is positively correlated with the presence of numerous resistant tumors, the mechanism of which may be modulated by the overexpression of resistance-related genes and proteins (27,28). We detected that the expression levels of p-ERK and STAT3 in K562/ADM cells were higher when compared with the levels in K562 cells. Meanwhile, knock-down of TRIB2 in normal K562/ADM cells resulted in the reduced activity of p-ERK and STAT3. To test our hypothesis, the ERK signal transduction pathway inhibitor U0126 was used to downregulate ERK phosphorylation. U0126 is an inhibitor of mitogen-activated protein kinase 1 and 2 (MEK1/2), which is known to downregulate ERK phosphorylation. U0126 is an inhibitor of the ERK signal transduction pathway. To test our hypothesis, phosphorylation of p-ERK and STAT3 in K562/ADM cells were higher when compared with that noted in the control group. While these initial findings are promising, more comprehensive and detailed studies need to be conducted, including in vivo animal models and more precise ERK1/2 pathway assays. Nevertheless, we demonstrated that decreased expression of resistant proteins is associated with inhibition of the ERK pathway through the knockdown of TRIB2, thereby reversing cell MDR.

Acknowledgements
The present study was supported by the National Natural Science Foundation (grant no. 31373121), the Shandong Science and Technology Committee (grant nos. ZR2014HQ079, ZR2014HLO56 and ZR2013HLO03), the Foundation of Shandong Educational Committee (grant nos. J17KA121 and J13LE11) and Young Backbone Teacher Development Support Project of Binzhou Medical University.

Competing interests
The authors declare no competing interests.

References