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Original Article

MicroRNA-424 overexpression attenuates high glucose and inflammation suppressed osteogenic differentiation

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Abstract: Objective: This study investigated the effects of high glucose (HG) and inflammation on osteogenic differentiation in human mesenchymal stem cells (HMSCs), and the regulatory effect of microRNA (miR)-424 on HGor inflammation-mediated osteogenic differentiation. Methods: HMSCs were cultured in five different differentiation-inducing mediums containing: (1) osteogenesis-inducing medium (OS) (control group), (2) OS + glucose, (3) OS + tumor necrosis factor (TNF), (4) OS + glucose + miR-424 antagonism, (5) OS + TNF + miR-424 antagonism, respectively. The expression levels of osteoblastic genes and miR-424 were determined by reverse transcription polymerase chain reaction (RT-PCR). The osteoblastic differentiation under HG condition and inflammation stimulation was observed by alizarin red S staining. Results: Compared with control group, *osteocalcin (OCN)* and *osteopontin (OPN)* expressions were lower, mineralized nodules appeared later, the total amount of mineralized nodules was smaller, the concentration of alizarin red was lower, and miR-424 expression was higher under HG condition and inflammation stimulation in experimental groups without miR-424 antagonism (all $P < 0.05$). After the addition of miR-424 antagonism, *OCN* and *OPN* expressions were up-regulated, formation of mineralized nodules advanced, total amount of mineralized nodules increased, and concentration of alizarin red was up-regulated compared to the experimental groups without miR-424 antagonism, but were still lower than the control group, while miR-424 expression was down-regulated compared to the experimental groups without miR-424 antagonism and the control group (all $P < 0.05$). Conclusion: MiR-424 might have a potential inhibitory role in HG or inflammation-mediated osteogenic differentiation.

Keywords: Mesenchymal stem cell, osteogenic differentiation, high glucose, inflammation, MiR-424 antagonism, osteoblast-related gene, mineralized nodule

Introduction

Mesenchymal stem cells (MSCs), also referred to multipotent stromal cells, are commonly isolated from various adult tissue sources, with adult bone marrow as one of the richest sources of MSCs [1]. MSCs are capable of undergoing multilineage differentiation into a variety of cell types, including osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells) and adipocytes (fat cells) [2]. Therefore, MSCs hold great hope for acting as excellent candidates for cell-based therapeutic strategies to regenerate injured or damaged tissues [3]. Furthermore, MSCs are readily accessible, easily expandable, and possess the potential to differentiate into mesoderm-derived lineages, leading to their suitability particularly for the application in skeletal tissue engineering strategies [4]. Regulation of differentiation of MSCs into osteogenic lineage is vital for the maintaince of bone mass and is necessary for the prevention of bone related diseases [5]. Currently, growing reports have evidenced that osteoblast differentiation is tightly controlled by several regulators including microRNA (miRNAs), which have the potential to regulate several gene expressions during the differentiation of MSCs towards osteoblastic cells, contributing to bone formation [6, 7].

MiRNAs comprise a group of endogenously expressed small noncoding RNA molecules (17-25 nt), which are involved in posttranscriptional regulation of their targets through protein transcription repression or mRNA degradation, and have been shown to have important roles in diverse biological processes [8]. MiRNAs have

been documented to act as important regulators in many cellular processes consisting of proliferation, development, differentiation, apoptosis, and cancer pathogenesis [9]. To date, more than 3% of the humans genes have been detected to encode miRNAs, and up to 40-90% of the human protein encoding genes are under miRNA-mediated gene regulation [10]. Several studies have identified populations of miRNAs in embryonic stem cells, hematopoietic stem cells, and multipotent mesenchymal stromal cells, as well as in their differentiated progeny, such as osteogenic differentiation [11, 12]. Moreover, a present study, which investigated a selective number of miRNAs for their expression and intracellular regulatory networks involved in differentiation in human mesenchymal stem cells (HMSCs) toward osteoblasts, has observed that miR-424 expression is specific in HMSCs [13]. Additionally, growing evidences suggested that high glucose (HG) has the ability to mediate the suppression of osteogenic differentiation in HMSCs, and proinflammatory cytokines are able to inhibit osteogenic differentiation and bone formation [14, 15].

In our present study, we aimed to investigate the effects of HG and inflammation on the osteogenic differentiation in HMSCs, and the regulatory effect of miR-424 on the HG or inflammation-mediated osteogenic differentiation in HMSCs.

Materials and methods

HMSC differentiation-inducing culture

HMSCs (SX) were purchased from Sxbio Biotechnology Co. (Shanghai, China). HMSCs in the second to fourth passage from amplification in vitro were successively cultured in five 4-well plates (5 groups) with cell density of 1×10^4/well. At day 2, the control group was added with an osteogenesis-inducing medium (OS) that contained dexamethasone (1×10^{-8} mol/L), vitamin C (0.05 g/L), β-glycerophosphate (β-GP, 0.01 mol/L) and bone morphogenetic protein 2 (BMP-2, 100 μg/L). HMSCs in the four experimental groups were cultured in different differentiation-inducing mediums containing: (1) OS + glucose (25 mmol/L), (2) OS + tumor necrosis factor (TNF, 10 ng/mL), (3) OS + glucose (25 mmol/L) + microRNA (miR)-424 antagonism, (4) OS + TNF (10 ng/mL) + miR-424 antagonism, respectively. The medium was changed every other day, meanwhile added with cytokine and/or treated with additional transfection. After 28 days, HMSCs were stained with alizarin red.

Reverse transcription polymerase chain reaction (RT-PCR)

HMSCs (1×10^4/well) were cultured in five 6-well plates (5 groups). Seven days after induction, HMSCs were digested and collected by using 0.25% pancreatin; and RNA was extracted. With an equal amount of RNAas template, complementary deoxyribonucleic acid (cDNA) was synthesized by RT; and the expression levels of osteoblastic gene including *osteocalcin (OCN)* and *osteopontin (OPN)* by RT-PCR. The primer sequences for *OCN* were as follows: forward, 5'-GACTGTGACGAGTTGGCTGAGG-3'; reverse, 5'-CTGGAGAGGAGCAGAACTGG-3'. The primer sequences for *OPN* were: forward, 5'-TTGCAGTGATTTGCTTTTGC-3'; reverse, 5'-GCCACAGCATCTGGGTAG-3'. The primer sequences for β-actin were: forward, 5'-CTGGCACCCAGCACAATG-3'; reverse, 5'-CCGATCCACACGGAGTACGGT-3'.

Alizarin red S staining

After the induction of osteogenic differentiation of HMSCs, the culture medium was removed. Then, HMSCs in 5 groups were washed by Dulbecco's phosphate-buffered saline (DPBS) three times, separately, and fixed with 75% ethanol for 30 min, followed by washing with distilled water three times. These fixed HMSCs were stained with 0.1% alizarin red-Tris-HCl (PH = 8.2) at 37°C for an hour. After staining, HMSCs were rapidly washed by 1% acetate solution and dehydrated by different concentrations of ethanol successively. Under a microscope, the stained HMSCs were photographed and then the degree of osteogenic differentiation was analyzed. HMSCs were decolorized by 5% sodium dodecyl sulphate-hydrochloride (SDS-HCl) for an hour, and the supernatant was aspirated, which was followed by the measurement of absorbance (OD) value at 405-nm wavelength. According to the OD values and the standard curve of alizarin red, the concentration of alizarin red in each group was calculated.

Detection of miR-424

The miR-424 was reverse transcribed by utilizing miR-424-specific stem-loop primer. The flu-

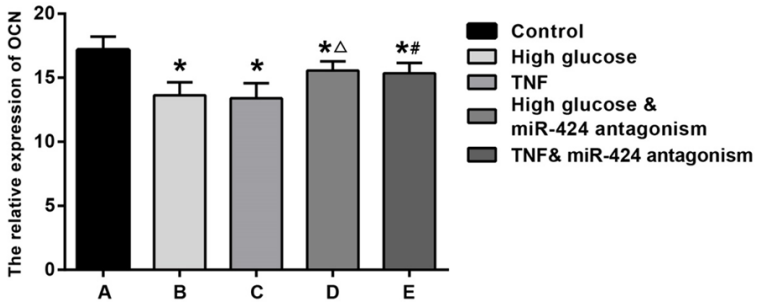

Figure 1. *OCN* gene expression levels in HBMCs cultured in different differentiation-inducing medium. Note: *OCN*, *osteocalcin*; HBMCs, human bone marrow cells; *, compared with A, $P < 0.05$; △, B vs. D, $P < 0.05$; #, C vs. E, $P < 0.05$.

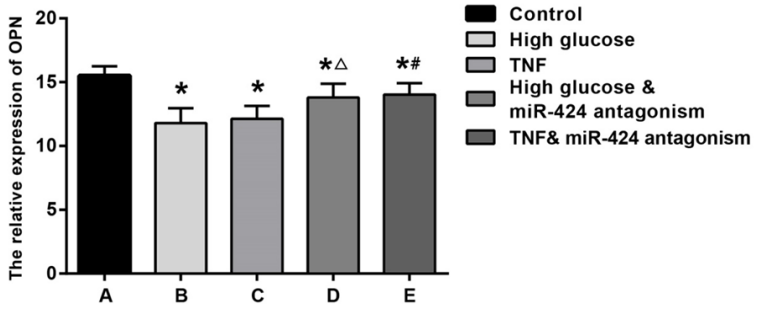

Figure 2. *OPN* gene expression levels in HBMCs cultured in different differentiation-inducing medium. Note: *OPN*: *osteopontin*; HBMCs, human bone marrow cells; *, compared with A, $P < 0.05$; △, B vs. D, $P < 0.05$; #, C vs. E, $P < 0.05$.

orescence quantitative PCR was performed with U6 as the internal reference. The relative abundance of miR-424 was calculated. The corresponding primer sequences were as follows: stem-loop: forward 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTCAAA-3', reverse 5'-GTGCAGGGTCCGAGGT-3'; U6: forward 5'-AACGCTTCACGAATTTGCGT-3', reverse 5'-AACGCTTCACGAATTTGCGT-3'.

Statistical analysis

The SPSS 18.0 software (IBM Corporation, Somers, NY, USA) was applied for statistical analysis. All measurement data were presented as mean ± standard (SD) and compared by using the *t* test or variance analysis. Enumeration data were presented as percentage or rate, and compared by the chi-square test. All *P* values were two-tailed, and a *P* value < 0.05 was considered statistically significant.

Results

Osteoblast-related gene expression

The levels of osteoblast-related gene expressions were demonstrated in **Figures 1** and **2**. As shown in **Figure 1**, the gene expressions of *OCN* in the experimental groups treated with glucose (HG, 13.63 ± 1.01) and TNF (inflammation stimulation, 13.40 ± 1.17) were lower than that in the control group (17.18 ± 1.02, both $P < 0.05$). After the addition of miR-424 antagonism, the *OCN* gene expressions under HG condition (15.55 ± 0.72) and inflammation stimulation (15.34 ± 0.82) were still lower than that in the control group (17.18 ± 1.02, both $P < 0.05$); while apparently increased as compared with the experimental groups without miR-424 antagonism under HG condition and inflammation stimulation (HG, 15.55 ± 0.72 vs. 13.63 ± 1.01, $P = 0.021$; inflammation stimulation, 15.34 ± 0.82 vs. 13.40 ± 1.17, $P = 0.035$). As seen in **Figure 2**, the *OPN* gene expressions in the experimental groups under HG condition (11.79 ± 1.17) and inflammation stimulation (12.12 ± 1.02) without miR-424 antagonism were lower than that in the control group (15.51 ± 0.73, both $P < 0.05$). After the addition of miR-424 antagonism, the *OPN* gene expressions under HG condition (13.79 ± 1.08) and inflammation stimulation (14.02 ± 0.91) were still lower than that in the control group (15.51 ± 0.73, both $P < 0.05$); while increased evidently when compared with the experimental groups without miR-424 antagonism under HG condition and inflammation stimulation (HG, 13.79 ± 1.08 vs. 11.79 ± 1.17, $P = 0.046$; inflammation stimulation, 14.02 ± 0.91 vs. 12.12 ± 1.02, $P = 0.032$).

Detection of mineralized nodules

Under the microscope, HMSCs grew adhering to the wall; initially, HMSCs showed long fusi-

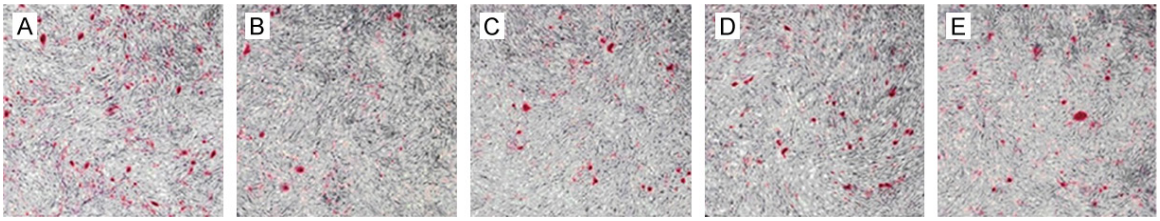

Figure 3. A microscope observation for the formation of mineralized nodules in HBMCs by alizarin red S staining (×100). Note: HBMCs, human bone marrow cells. Note: A. HMSCs were cultured in osteogenesis-inducing medium (OS) (control group); B. HMSCs were cultured in a differentiation-inducing medium containing OS + glucose; C. HMSCs were cultured in a differentiation-inducing medium containing OS + tumor necrosis factor (TNF); D. HMSCs were cultured in a differentiation-inducing medium containing OS + TNF + miR-424 antagonism; E. HMSCs were cultured in a differentiation-inducing medium containing OS + glucose + miR-424 antagonism; miR-42, microRNA-424.

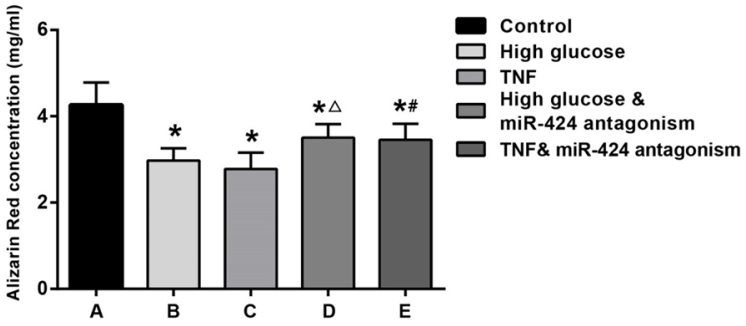

Figure 4. Concentration of alizarin red in HBMCs cultured in different differentiation-inducing medium. Note: HBMCs, human bone marrow cells; *, compared with A, $P < 0.05$; △, B vs. D, $P < 0.05$; #, C vs. E, $P < 0.05$.

form shape, large volume, smooth edge and plumpness; with cell density increased gradually, HMSCs were aggregated, presenting a multilayer growth locally, and forming an eddy-like distribution; at day 12, brown nodules appeared, and the amount of mineralized nodules increased gradually in the control group (**Figure 3A**). In the experimental groups, both eddy-like distribution and mineralized nodules appeared later, and the total amount of mineralized nodules was smaller as compared with the control group (**Figure 3B**, **3C**). Under HG condition and inflammation stimulation, the formation of eddy-like distribution and the appearance of mineralized nodules in the experimental groups with miR-424 antagonism advanced, and the total amount of mineralized nodules in the experimental groups with miR-424 antagonism increased, compared with the experimental groups without miR-424 antagonism (**Figure 3D**, **3E**). After decoloration with 5% SDS-HCl, the measurement of the concentration of alizarin red under different culture conditions showed varied results, which were in consistent with the observation results under the microscope (**Figure 4**). The concentration of alizarin red in the experimental groups treated with glucose (HG condition, 2.97 ± 0.29) and TNF (inflammation stimulation, 2.78 ± 0.38) were lower than that in the control group (4.26 ± 0.52, both $P < 0.05$). After the addition of miR-424 antagonism, the concentration of alizarin red under HG condition (3.50 ± 0.32) and inflammation stimulation (3.45 ± 0.38) were still lower than that in the control group (4.26 ± 0.52, both $P < 0.05$); while were up-regulated observably when compared with the experimental group without miR-424 antagonism (HG, 3.50 ± 0.32 vs. 2.97 ± 0.29, $P = 0.0495$; inflammation stimulation, 3.45 ± 0.38 vs. 2.78 ± 0.38, $P = 0.047$).

Associations between miR-424 expression and osteogenic differentiation

The expression levels of miR-424 under different culture conditions were displayed in **Figure 5**. Compared with the control group, the expression levels of miR-424 in the experimental groups without miR-424 antagonism under HG condition and inflammation stimulation were higher (0.97 ± 0.12 vs. 1.26 ± 0.18, 0.97 ± 0.12 vs. 1.42 ± 0.29, both $P < 0.05$); while the expression levels of miR-424 in the experimental groups added with miR-424 antagonism were lower under HG condition and inflammation stimulation (0.97 ± 0.12 vs. 0.73 ± 0.07, 0.97 ± 0.12 vs. 0.74 ± 0.10, both $P < 0.05$). Under HG condition and inflammation stimula-

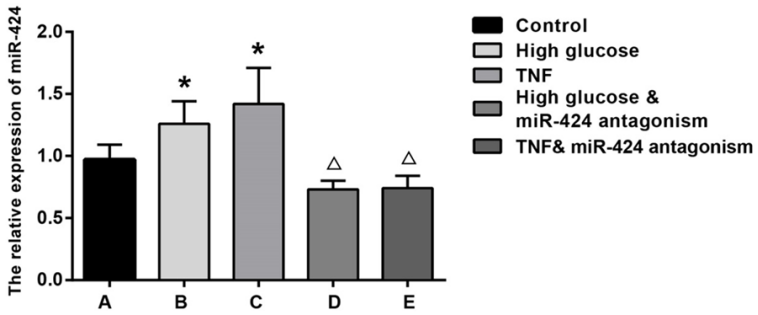

Figure 5. Expression levels of miR-424 in HBMCs cultured in different differentiation-inducing medium. Note: miR-42, microRNA-424; HBMCs, human bone marrow cells; *, compared with A, $P < 0.05$; △, B vs. D. $P < 0.05$; #, C vs. E, $P < 0.05$.

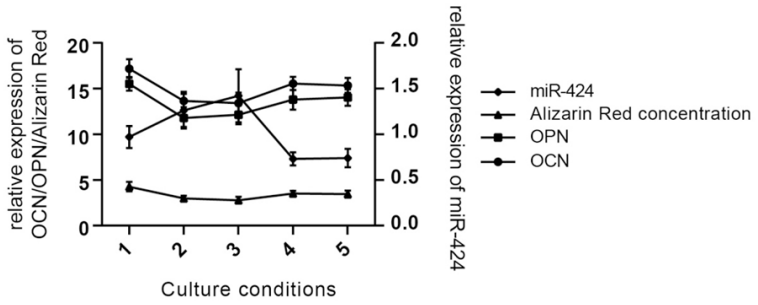

Figure 6. Associations between miR-424 expression levels and osteogenic differentiation under different culture conditions. Note: miR-424, microRNA-424; 1, control group; 2, experimental group treated with osteogenesis-inducing medium (OS) + glucose (25 mmol/L); 3, experimental group treated with OS + tumor necrosis factor (TNF, 10 ng/mL); 4, experimental group treated with OS + TNF (10 ng/mL) + miR-424 antagonism; 5, experimental group treated with OS + glucose (25 mmol/L) + miR-424 antagonism.

tion, the expression levels of miR-424 in the experimental groups added with miR-424 antagonism were up-regulated significantly as compared with the experimental group without miR-424 antagonism (HG, 1.26 ± 0.18 vs. 0.73 ± 0.07, $P = 0.002$; 1.42 ± 0.29 vs. 0.74 ± 0.10, $P = 0.004$). As presented in **Figure 6**, under HG condition and inflammation stimulation, miR-424 expression levels increased, while osteoblastic genes (*OCN* and *OPN*) expression levels and the concentration of alizarin red decreased; after added with miR-424 antagonism, the miR-424 expression levels decreased to lower than that in the control group, while the expression levels of osteoblastic genes including *OCN* and *OPN* and the concentration of alizarin red increased, which were still lower than those in the control group. These results revealed that miR-424 expression level was negatively associated with the expression levels of osteoblastic genes including *OCN* and *OPN* and the amount of mineralized nodules. During osteogenic differentiation, miR-424 might act as an inhibitory regulator.

Discussion

In our present study, by using PT-PCR, we found that HG and inflammation stimulation might be associated with the down-regulation of the expression levels of osteoblast-related genes including *OCN* and *OPN*, and the expression levels of *OCN* and *OPN* gene were further up-regulated as miR-424 expression decreased which was mediated viamiR-424 antagonism. The up-regulation of *OCN* and *OPN* gene expression bymiR-424 antagonism under HG condition and inflammation stimulation still could not reach the levels in the control group. In addition, our alizarin red S staining revealed that HG and inflammation stimulation might be negatively related to mineralized nodule formation in HMSCs, while down-regulated miR-424 via miR-424 antagonism might be positively related to the formation of mineralized nodules in HMSCs. These findings suggested that HG and inflammation stimulation might result in inhibiting osteogenic differentiation in HMSCs, while down-regulated miR-424 might have the ability to promote osteogenic differentiation in HMSCs under HG condition and inflammation stimulation. It has been documented that glucose in the microenvironment is capable of markedly influencing proliferation, regulation, differentiation, and cell apoptosis and senescence [16]. In rat MSCs, it has been evidenced that the decrease of glucose stimulated cell proliferation while HG enhanced cell apoptosis [17]. Impairment of regenerative proliferation and differentiation capacity in MSCs may result from a decrease of the stem cell pool, an

impairment of transient amplifying segment after the division of asymmetric stem cell, and functional defects of their offspring after maturation, which resulted from HG [18]. In line with our results, many previous studies have identified the mediating role of HG in the suppression of osteogenic differentiation [14, 19, 20]. In addition, in bone metabolism, a number of inflammatory cytokines, such as TNF-α, have been evidenced in promoting bone loss through the activation of osteoclastogenesis and the reduction of bone mineral density through inhibiting osteogenic differentiation as well as bone formation [21]. In consistent with our results, a previous review has illuminated the fact that the inflammatory cascade affects osteogenic differentiation in MSCs [22].

Furthermore, we observed that HMSCs under HG condition and inflammation stimulation had higher miR-424 expression than HMSCs in the control group. After added with miR-424 antagonism, the expression level of miR-424 in HMSCs was greatly down-regulated under HG condition and inflammation stimulation. The analysis of the associations of miR-424 with osteogenic differentiation demonstrated that HG and inflammation stimulation up-regulated the expression level of miR-424 while down-regulated the expression level of *OCN* and *OPN* gene and the concentration of alizarin red in HMSCs, while miR-424 antagonism could reverse these results. These results indicated that HG and inflammation stimulation might serve as inhibitors in osteogenic differentiation, through mediating miR-424 expression inhibiting *OCN* and *OPN* gene expression and mineralized nodules formation. Here, it was presented that the decreased osteogenic differentiation in HMSCs in HG and inflammation-stimulating media is accompanied with a down-regulation of miR-424. As expressed ectopically, miR-424 restores osteoblast mineralization and viability under HG condition and inflammation stimulation. We hypothesized that the underlying mechanism is that miR-424 post-transcriptionally regulated its targets, *OCN* and *OPN* gene, through protein transcription repression or mRNA degradation, promoting osteogenic differentiation [23]. Moreover, a number of miRNAs have been identified to play crucial roles in osteogenic differentiation under HG condition or inflammation stimulation, such as You et al. have suggested that the overexpression of miR-378 attenuates HG-suppressed osteogenic differentiation by targeting *caspase-3* and activating the phosphatidylinositol 3-kinase (PI3K)/Akt pathway [24]. Additionally, Sol Kim et al. indicated the regulatory role of miR-103a-3p in the osteogenic differentiation inhuman adipose derived stem cells (HADSCs) and proliferation of HADSCs by directly targeting of *cyclin-dependent kinase 6* and *dicer1* gene partly [25].

In conclusion, our present study demonstrates that HG and inflammation stimulation may play negative roles in regulating osteogenic differentiation in HMSCs. In addition, a potential regulatory role of miR-424 is revealed in osteogenic differentiation of HMSCs in vitro through mediating *OCN* and *OPN* gene expression, and inhibition of miR-424 may contribute to repair osteogenic differentiation. These results further provide an alternative strategy for improving the efficiency of osteogenic differentiation. However, additional investigations are needed to clarify its function in vivo.

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Disclosure of conflict of interest

None.

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