S-sulfhydration of MEK1 leads to PARP-1 activation and DNA damage repair

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Abstract

The repair of DNA damage is fundamental to normal cell development and replication. Hydrogen sulfide (H₂S) is a novel gasotransmitter that has been reported to protect cellular aging. Here, we show that H₂S attenuates DNA damage in human endothelial cells and fibroblasts by S-sulfhydrating MEK1 at cysteine 341, which leads to PARP-1 activation. H₂S-induced MEK1 S-sulfhydration facilitates the translocation of phosphorylated ERK1/2 into nucleus, where it activates PARP-1 through direct interaction. Mutation of MEK1 cysteine 341 inhibits ERK phosphorylation and PARP-1 activation. In the presence of H₂S, activated PARP-1 recruits XRCC1 and DNA ligase III to DNA breaks to mediate DNA damage repair, and cells are protected from senescence.

Keywords H₂S; MEK1; PARP-1; S-sulfhydration

Subject Categories Post-translational Modifications, Proteolysis and Proteomics; DNA Replication, Repair and Recombination

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Introduction

Hydrogen sulfide (H₂S) has been recognized as one of the important gasotransmitters together with nitric oxide (NO) and carbon monoxide in mammals [1–3]. Cystathionine gamma-lyase (CSE), cystathionine beta-synthase and 3-mercaptopyruvate sulfurtransferase are the three enzymes responsible for the endogenous H₂S production [2]. The expressions of these enzymes are tissue-specific. Compared with all other tissues in the body, vascular tissues express abundant CSE proteins and produce a large amount of H₂S [1,4,5]. Deficiency of CSE reduces H₂S production in vascular tissues and leads to endothelial dysfunction and high blood pressure in an age-dependent manner in mice [1]. H₂S has been recently characterized as both endothelium-derived relaxing factor and endothelium-derived hyperpolarizing factor [6,7]. S-sulfhydration is proposed to mediate most of these effects from H₂S by yielding a hydropersulfide moiety (–SSH) in cysteine residues of targeted proteins [8].

The integrity of endothelial cell function is compromised with aging, and impaired DNA damage repair plays pivotal roles in the evolution of age-associated cardiovascular disorders [9]. The salvage of DNA damage is fundamental to normal cell development and biological functions [10]. DNA damage evokes a complex and highly coordinated DNA damage repair response that is integral to the maintenance of genomic stability [11]. Poly(ADP-ribose)ation, carried out by poly(ADP-ribose)ation polymerases (PARPs), is one of the earliest and major cellular responses to DNA damage repair. PARPs transfer ADP-ribose from NAD⁺ to glutamic acid residues on a protein acceptor or itself, allowing the formation of ADP-ribose polymers (PARs) [12]. Upon DNA damage, PARPs bind to DNA strand breaks and catalyze the addition of long branched chains of PARs onto itself and other chromatin remodeling factors [12,13]. As a result of self-poly(ADP-ribose)ation, other DNA damage repair proteins, e.g., XRCC1, pol β and DNA ligase IIIα, are recruited to the sites of DNA breaks and repair DNA damage [13]. The activation of PARPs is tightly regulated by a series of kinases, in which MEK/ERK pathway plays a critical role in initiating PARP automodification [13–15].

We had recently demonstrated that deficiency of CSE leads to early development of cellular senescence in mouse embryonic fibroblasts (MEFs) [16]. Here, we observed that H₂S increases PARP-1 activity and attenuates DNA damage in human endothelial cells and fibroblasts. More DNA damage occurred in CSE-deficient mouse embryonic fibroblasts. Moreover, H₂S S-sulfhydrated MEK1 at cysteine 341 and induced ERK1/2 phosphorylation, which subsequently translocates into nucleus and leads to PARP-1 activation and improved DNA damage repair and cellular senescence. Mutation of cysteine 341 in MEK1 diminished H₂S-induced ERK phosphorylation and PARP-1 activation.

Results and Discussion

H₂S induces PARP-1 activity

PARP-1 is an abundant nuclear protein and plays an important role in DNA damage repair. PARP-1 activity can be reflected by PAR levels in cells or tissues [17]. We first tested the impact of NaHS (a well-known H₂S donor) on PARP-1 activity at lower concentration

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(0.1–10 μ M) in human umbilical vein endothelial cells (HUVECs). NaHS treatment increased PAR level in a dose-dependent manner in HUVECs (Fig 1A). Liver and kidney are two main H₂S-producing organs in mammals [8,18]. H₂S production rate was decreased approximately by 80% in both liver and kidney tissues from CSE knockout (CSE-KO) mice in comparison with wild-type littermates (WT) [8,18]. Consistent with the data of endothelial cells, PAR levels were lower in CSE-KO kidney and liver tissues in comparison with their counterparts from WT mice (Fig 1B and C). Murine heart tissues were reported to have lower H₂S-producing potential, which is presumably due to loss of CSE translation despite the presence of CSE mRNA [19]. PAR level in heart tissues from both WT and CSE-KO mice was compared, and we found that PAR is hard to detect in heart tissues from both genotypes (Fig 1D). Similar to heart tissues, PARP-1 activity in skeletal muscle and spleen tissues from both genotypes was minimal (Supplementary Fig S1A and B). Compared with liver and kidney tissues from WT mice, CSE expression in skeletal muscle and spleen tissues was pretty weaker [20]. These data suggest that the level of PAR is correlated with the expression level of CSE. PAR levels in MEFs isolated from WT and CSE-KO mice were further compared, and we found significant decreased PAR levels in CSE-KO MEFs compared with WT MEFs (Fig 1E). Poly (ADP-ribose) glycohydrolase (PARG) is a major enzyme responsible for the catabolism of PAR [12]. We next measured PARG activity in liver, kidney and heart tissues from both WT and CSE-KO mice. Although PARG activity was positively correlated with PAR level, the lack of CSE did not affect PARG activity in all these three tissues (Supplementary Fig S1C), suggesting H₂S-stimulated PAR is not due to altered PARG activity. Higher level of PAR in kidney tissues and MEFs from WT mice observed here were unlikely due to the experimental operation with the cell exposure in the extraction buffer, because the supplement of PARP-1 inhibitor DPQ in the extraction buffer did not affect PAR level in kidney tissues and MEFs from WT mice; however, we did observe that DPQ inhibits PAR after WT MEFs are incubated with DPQ for 2 h *in vitro* (Supplementary Fig S2A and B).

Besides its role in DNA damage repair, PARP-1 has also been reported to be involved in cell apoptosis elicited by PAR-induced release of apoptosis-inducing factor (AIF) from mitochondria [21]. To investigate whether H₂S-stimulated PARP-1 activity induces cell apoptosis in HUVECs, we performed annexin V-FITC staining in the cells after H₂S treatment. We did not observe more apoptosis (FITC positive) in HUVECs after NaHS treatment as well as cell necrosis (propidium iodide positive, Supplementary Fig S3A). In addition, no more AIF release was detected from mitochondria in HUVECs treated with NaHS (Supplementary Fig S3B). This implies that PARP-1 activation by H₂S does not cause cell degeneration in HUVECs.

H₂S improves DNA damage repair by activating PARP-1

Considering the crucial role of PARP-1 in DNA damage repair response and the stimulatory role of H₂S in PARP-1 activation observed herein, we tested the effects of H₂S on DNA damage repair response. Methyl methanesulfonate (MMS), a well-known agent to induce single- and/or double-strand DNA breaks in mammalian cells, was applied here to induce DNA damage in HUVECs [22]. Firstly, we compared PAR level in HUVECs treated with MMS in the presence or absence of NaHS. NaHS activated PARP-1 activity at as

early as 5 min, and the supplement of MMS further strengthened NaHS-stimulated PARP-1 activity. MMS alone only activated PARP-1 activity at 30 min (Supplementary Fig S4A). To test the influence of H₂S-induced PARP-1 activation on DNA damage, two methods were applied here for assessing DNA damage. The first one is apurinic/apyrimidinic (AP) contents in genomic DNA, while the other is comet tail assay [23,24]. More AP contents and longer comet tail would demonstrate more DNA damage inside the cells [23,24]. We observed more DNA damage in HUVECs treated with MMS, which was significantly reversed by NaHS co-treatment (Fig 2A and B). We further found that the administration of DPQ deteriorates H₂S-restored DNA damage (Fig 2A and B) [12]. This clarifies the important role of PARP-1 activation in H₂S-attenuated DNA damage. Upon DNA damage, PARP-1 will recruit other DNA damage repair proteins, including XRCC1 and DNA ligase III, to repair DNA damage [13]. With co-immunoprecipitation, we further found that MMS induces the binding of PARP-1 with XRCC-1 and DNA ligase III in HUVECs, which is significantly enhanced in the presence of NaHS (Fig 2C), suggesting the stimulatory role of H₂S on the complex formation of PARP-1/XRCC-1/DNA ligase III.

Accumulated DNA damage is one important causative factor for cellular aging process [25]. MMS treatment induced more senescent cells, which was reversed by H₂S but strengthened by DPQ in HUVECs (Fig 2D). We previously reported that CSE-KO MEFs are easy to become senescent compared with WT MEFs [14]. We further found here that CSE-KO MEFs have less PARP-1 activity, but more DNA damage when compared with WT MEFs even in the absence of MMS (Supplementary Fig S4B). The expression levels of CSE mRNA and protein as well as H₂S production rate were significantly increased in HUVECs after MMS treatment (Supplementary Fig S4C, D and E), which probably represents one compensative response of anti-DNA damage in endothelial cells under stress condition. We further observed that NaHS stimulates PARP-1 activity and attenuates MMS-induced DNA damage in human fibroblasts (Supplementary Fig S5A and B). MMS also induced CSE expression in human fibroblasts (Supplementary Fig S5C). We previously showed that knockdown of CSE by siRNA significantly stimulates cellular senescence in human fibroblasts [16].

Involvement of ERK phosphorylation in H₂S-stimulated PARP-1 activity

To elucidate the mechanisms underlying H₂S stimulation of PARP-1 activity, we firstly tested whether H₂S could directly activate PARP-1. NaHS was incubated with purified human PARP-1 for half an hour, and then PAR was detected using Western blotting. NaHS treatment did not increase PAR levels at concentration of 0.01 to 1 mM (Supplementary Fig S6A). S-sulphydration has been identified as one novel post-translational modification by H₂S in eukaryotic cells, which is the addition of one sulphydryl to thiol side of cysteine residue and formation of persulfide group (R-S-S-H) [8,16]. Here, we investigated whether H₂S could directly S-sulphydrate PARP-1 using biotin-switch assay. Our result showed that H₂S does not S-sulphydrate either unactivated or activated PARP-1 in HUVECs, while GAPDH is clearly S-sulphydrated (Supplementary Fig S6B). These results indicate that H₂S does not interact with PARP-1 directly. It appears that there are some other pathways for H₂S-induced PARP-1 activation.

Figure 1. H₂S-augmented PARP-1 activity (PAR).

A H₂S-induced PAR level in HUVECs. The cells were incubated with NaHS at the indicated concentration for 2 h. * P = 0.0026; # P = 0.0002.

B, C Less PAR was found in kidney (* P = 0.031) and liver tissues (* P = 0.015) from CSE-KO mice when compared with those from WT mice.

D There was no difference in PAR level between WT heart and CSE-KO heart tissues.

E Lower PAR was detected in CSE-KO MEFs compared with WT MEFs.

Data information: All the data are from at least three independent experiments.

Source data are available online for this figure.

It has been reported that PARP-1 can be stimulated directly by interaction with phosphorylated ERK1/2 [14,15]. We detected the temporal change of phosphorylated ERK1/2 and PARP-1 activity in HUVECs with NaHS treatment. It was found that the levels of both PAR and phosphorylated ERK1/2 are increased by NaHS treatment (Fig 3A). The change of phosphorylated MEK1/2, which is upstream activator of ERK1/2, did not alter significantly by NaHS treatment (Fig 3A). U0126, an inhibitor of ERK1/2 phosphorylation by MEK1/2, was applied to determine the interaction between phosphorylated ERK1/2 and PARP-1 activation in HUVECs [14]. The results showed

U0126 decreases H₂S-induced PAR (Fig 3B). All these results indicate that the involvement of MEK1/2 in H₂S stimulates ERK1/2 phosphorylation and PARP-1 activity. It is worthy to note here that H₂S-induced activation of ERK1/2 is not through MEK1/2 phosphorylation because NaHS has no effect on MEK1/2 phosphorylation (Fig 3A). U0126 was further applied to test the relationship between ERK phosphorylation and DNA damage in HUVECs. We observed that U0126 reverses NaHS-protected DNA damage in the presence of MMS (Fig 3C). PD98059, another inhibitor of MEK/ERK, displayed similar effects as U0126 (Supplementary Fig S7A and B) [15,26].

Figure 2. Mediation of PARP-1 in H₂S-protected DNA damage repair.

A H₂S reduced AP contents of genomic DNA in HUVECs. **P* = 0.0015; #*P* = 0.0028; ***P* = 0.0355. The data were from four independent experiments.

B H₂S decreased comet tail length. The data were from three independent assays, and more than 30 random comet tails were calculated. **P* = 9.11787E-27; #*P* = 2.24E-10; ***P* = 1.82E-09. Bar = 50 μm in all images.

C H₂S stimulated the formation of PARP-1–XRCC-1–Ligase III complex. The data were from three independent assays.

D H₂S reduced cellular senescence in HUVECs. For each assay, more than 4 continual fields were analyzed and 1000 cells were counted for senescence assay. **P* = 6.63943E-06; #*P* = 4.73237E-06; ***P* = 0.001. Bar = 50 μm in all images.

Data information: In all studies, HUVECs were treated with 1 mM MMS, 10 μM NaHS and/or 10 μM DPQ for 2 h.

Source data are available online for this figure.

siRNA-mediated knockdown of MEK1 or ERK1/2 also prevented NaHS-induced PARP-1 activity and attenuated NaHS-protected DNA repair (Supplementary Fig S7C, D and E). All these data suggest the critical role of ERK activation in the protective effect of H₂S against DNA damage.

To elucidate the involvement of MEK/ERK in the activating process of PARP-1 by H₂S, we detected the interaction between ERK1/2 and PARP-1. Co-immunoprecipitation data showed that more phosphorylated forms of ERK1/2 interact with PARP-1 after NaHS treatment (Fig 3D). Distribution of phosphorylated ERK1/2 was compared in both nuclear and cytosol part of HUVECs after

NaHS treatment. We observed there is more phosphorylated ERK1/2 in nuclear fraction in NaHS-treated HUVECs compared with control cells, while similar amount of phosphorylated ERK1/2 was found in cytosols (Fig 3E). This signifies that more phosphorylated forms of ERK1/2 translocate into the nucleus and interact with PARP-1 after H₂S treatment in HUVECs.

H₂S post-translationally modifies MEK1 via S-sulphylation

As inhibition of MEK1/2 decreases the phosphorylation of ERK1/2 and PARP-1 activity, we next investigated whether H₂S could

Figure 3. H₂S activated PARP-1 through MEK/ERK pathway.

- A** Temporal change of PARP-1 activity and phosphorylation of ERK1/2 and MEK1/2 by H₂S. HUVECs were treated with 10 μM NaHS for the indicated time. p, phosphorylated; t, total.
- B** U0126 reversed H₂S-stimulated PARP-1 activation in HUVECs. The cells were incubated with U0126 (10 μM) in the presence or absence of NaHS (10 μM) for 2 h. **P* = 0.018 versus NaHS group.
- C** U0126 reversed H₂S-protected DNA damage. HUVECs were incubated with U0126 (10 μM) in the presence or absence of MMS (1 mM) and/or NaHS (10 μM) for 2 h. **P* = 5.07116E-09 versus MMS group; #*P* = 1.55E-09 versus MMS plus NaHS group. For each group, more than 30 random comet tails were assayed.
- D** H₂S stimulated the binding of phosphorylated ERK1/2 with PARP-1. HUVECs were incubated with NaHS (10 μM) for 2 h.
- E** H₂S induced phosphorylated ERK1/2 translocation into nucleus. HUVECs were incubated with NaHS (10 μM) for 2 h. **P* = 0.001786; #*P* = 6.57978E-05.

Data information: All the data are from at least three independent experiments.
Source data are available online for this figure.

directly modify MEK1/2. After 2-h treatment with 10 μM NaHS, HUVECs were collected and subjected to biotin-switch assay. GAPDH was selected as a positive control, which is known to be S-sulphydrated by H₂S [8]. We found that MEK1/2 is basically S-sulphydrated, and NaHS treatment further increases MEK1/2 S-sulphydration in HUVECs (Fig 4A). Classically, RAS is localized in the upstream of MEK/ERK activation cascade (RAS–RAF–MEK–ERK cascade), and RAS has been reported to be S-nitrosylated by NO at

cysteine residue 118 [26]. Besides, ERK1/2 can be S-nitrosylated by NO as well [27]. However, neither RAS nor ERK1/2 could be S-sulphydrated after NaHS incubation (Fig 4A). And next total protein of kidney tissues from both WT and CSE-KO mice was used for biotin-switch assay as well. WT mouse kidney displayed more S-sulphydrated MEK1/2 in comparison with that from CSE-KO mice (Supplementary Fig S8A). As MEK1 and MEK2 share higher identity for their amino acid sequences (80%), it seems reasonable to infer

Figure 4. H₂S S-sulphydrated MEK1 at cysteine residue 341 and facilitated ERK phosphorylation.

- A H₂S S-sulphydrated MEK1/2 but not RAS and ERK1/2 in HUVECs. The cells were incubated with NaHS (10 μM) for 2 h and subjected to biotin-switch assay with antibodies against MEK1/2, RAS, ERK1/2 and GAPDH, respectively.
- B H₂S S-sulphydrated MEK1 but not MEK2 in HEK293 cells. The cells were incubated with NaHS (10 μM) for 2 h and subjected to biotin-switch assay with antibodies against MEK1 and MEK2, respectively.
- C H₂S S-sulphydrated MEK1/2 in HEK293 cells.
- D H₂S S-sulphydrated MEK1 at cysteine residue 341.
- E MMS enhanced MEK1 S-sulphydration.
- F H₂S stimulated ERK2 phosphorylation in the presence of MEK1.
- G Mutation of cysteine 341 in MEK1 diminished H₂S-induced activation of ERK2.
- H Mutation of cysteine 341 reduced H₂S-induced PAR level.

Data information: In (C), (D), (E) and (H), HEK293 cells were first transfected with His6-MEK1 or His6-MEK1 cysteine residue mutants for 48 h, the cells were then incubated with NaHS (10 μM) with or without MMS (1 mM) for 2 h. In (F) and (G), HEK293 cells were first transfected with His6-MEK1 or His6-MEK1 cysteine residue mutants together with GFP/ERK2 for 48 h, and the cells were then incubated with NaHS (10 μM) for 2 h. All the data were from at least three independent experiments. Source data are available online for this figure.

that both MEK1 and MEK2 can be modified by H₂S. Using a MEK1- or MEK2-specific antibody, we observed that MEK1 but not MEK2 was S-sulfhydrated by NaHS in HUVECs (Fig 4B). We further observed that NaHS also S-sulfhydrates MEK1 in human fibroblasts (Supplementary Fig S8B).

We next constructed His6-tagged MEK1 cDNA plasmids and transfected into HEK293 cells, from which total protein was isolated and subjected to biotin-switch assay. As expected, we observed more S-sulfhydrated His6-MEK1 after NaHS treatment (Fig 4C). To investigate the molecular mechanism of MEK1 S-sulfhydration, all the six cysteine residues in MEK1 were site-mutated to glycines, including cysteine 121, 141, 207, 277, 341 and 376 [28]. No S-sulfhydrated His6-MEK1 was observed only when cysteine 341 was mutated, while MEK1 S-sulfhydration still existed in all other mutants (Fig 4D). MMS enhanced MEK1 S-sulfhydration in His6-MEK1-transfected cells, but failed to do so when cysteine 341 was mutated (Fig 4E). We further found that CSE overexpression induces MEK1 S-sulfhydration in HEK-293 cells (Supplementary Fig S8C). To study whether cysteine 341 in MEK1 is also responsible for H₂S-stimulated ERK1/2 phosphorylation, MEK1 cDNA and GFP-ERK2 were co-transfected into HEK293 cells for 48 h [29]. Then, the cells were treated with NaHS for 2 h and harvested. In control or NaHS-treated HEK293 cells only carrying GFP-ERK2, we did not observe any phosphorylation of GFP-ERK2. Thus, ERK2 is unlikely a direct target of NaHS (Fig 4F). When MEK1 was co-transfected with ERK2, phosphorylation of ERK2 was observed. Furthermore, the administration of NaHS enhanced more phosphorylated GFP-ERK2 in HEK293 cells (Fig 4F). Mutation of cysteine 341 but not cysteine 277 in MEK1 eliminated ERK2 phosphorylation even in the presence of H₂S (Fig 4G). Lower PAR level was observed in HEK293 cells transfected with MEK1 C341G compared with those transfected with MEK1 C277G and wild-type MEK1 when the cells were treated with NaHS (Fig 4H). From these data, it can be postulated that cysteine 341 is critical for MEK1 activating ERK2 and PARP-1 activation.

As binding of ERK2 with MEK1 is essential for the activation of ERK2 phosphorylation [30], we tested the binding affinity of MEK1, MEK1 C277G, MEK1 C341G with GFP-ERK2. MEK1 C341G displayed similar binding affinity to GFP-ERK2 as MEK1 and MEK1 C277G, which implies that cysteine 341 in MEK1 is not involved in ERK binding (Supplementary Fig S8D). H₂S S-sulfhydration of cysteine 341 may elicit a conformational change of catalytic domain in MEK1, which would consequently induce the activity of MEK1. More structural investigation will be needed to address how cysteine 341 contributes to H₂S-regulated MEK1 activity.

In summary, we present a novel physiological aspect of H₂S as a DNA damage protectant via a signal cascade, which is H₂S S-sulfhydrating and activating MEK1 at cysteine 341, followed by phosphorylated ERK1/2 translocation into nucleus in stimulating PARP-1 activity through direct interaction (Fig 5). For the first time, our results clarify the role of endogenous H₂S in strengthening DNA damage repair process through targeting at DNA damage repair protein PARP-1. Genetic instability due to increased DNA damage has an important impact on the pathogenesis of human diseases. Abnormal DNA damage repair interferes with fundamental cell features such as cell differentiation, cell cycle regulation and apoptosis. Deficiency of H₂S is observed in various pathological conditions, including hypertension, atherosclerosis, cancers, Alzheimer's disease, gastric mucosal injury, liver cirrhosis and

Figure 5. Signal cascade of H₂S stimulating PARP-1 activity and DNA damage repair.

Post-translational modification symbols are italicized. p, phosphorylation; SSH, S-sulfhydration; PAR, poly(ADP-ribosylation).

aging [2,5]. The present discovery further suggests that targeting at CSE/H₂S system would be a new therapeutic avenue for the treatment of DNA-damage-related diseases, and even for human life extension.

Materials and Methods

Mek1 gene cloning and site mutagenesis

Total RNA was isolated from HUVECs using the RNeasy mini kit (Qiagen, Hilden, Germany). One microgram of total RNA and oligo-d(T) primer was used for reverse transcriptase reaction with the AMV First Strand cDNA Synthesis kit (New England Biolabs Inc., Ipswich, MA). Digested MEK1 and pCDNA3.1/myc-his6 (Life technologies, Burlington, ON) with HindIII and BamHI were purified from agarose gel and then linked with T4 ligase (Promega, Madison, WI) for 1 h at room temperature. Single or double mutation was conducted using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) [16]. All primer sequences were listed in the Supplementary Table S1.

Biotin-switch assay

S-sulfhydration assay was performed as described previously [7,8,16]. Anti-GAPDH antibody was applied as positive control for the assay [8]. Please see Supplementary Materials and Methods for detail.

Western blotting, immunoprecipitation and quantitative RT-PCR

See Supplementary Materials and Methods for detail.

RNA interference

Pre-designed MEK1 and ERK1/2 siRNA were purchased from Cell Signaling technology Inc. (Beverly, MA). HUVECs were grown to more than 80% confluence and followed by siRNA transfection using transfection reagents from Santa Cruz (Dallas, TX) for 48 h prior to cell collection.

AP site quantification assay

Genomic DNA of HUVECs with different treatments was isolated according to the instructions of OxiSelect Oxidative DNA damage Quantification kit (Cell Biolabs, INC, San Diego, CA).

Comet tail assay and senescent beta-galactosidase staining

See Supplementary Materials and Methods for detail.

Statistical analysis

All quantitative data were all presented by mean $\pm$ standard error. Significance comparison between two groups was made with Student's *t*-test with two-tailed distribution. Statistical significance was determined by *P*-value < 0.05 .

Supplementary information for this article is available online: <http://embor.embopress.org>

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Author contributions

KZ, RW and GY designed the study; KZ, YJ, ZA, SL and GY acquired the data; KZ and GY analyzed and interpreted the data; KZ and GY wrote and revised the manuscript. All authors discussed the results and commented on the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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