Enhanced vulnerability to oxidative stress and induction of inflammatory gene expression in 3-phosphoglycerate dehydrogenase-deficient fibroblasts

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L-Serine (L-Ser) is a necessary precursor for the synthesis of proteins, lipids, glycine, cysteine, D-serine, and tetrahydrofolate metabolites. Low L-Ser availability activates stress responses and cell death; however, the underlying molecular mechanisms remain unclear. L-Ser is synthesized de novo from 3-phosphoglycerate with 3-phosphoglycerate dehydrogenase (Phgdh) catalyzing the first reaction step. Here, we show that L-Ser depletion raises intracellular H2O2 levels and enhances vulnerability to oxidative stress in Phgdh-deficient mouse embryonic fibroblasts. These changes were associated with reduced total glutathione levels. Moreover, levels of the inflammatory markers thioredoxin-interacting protein and prostaglandin-endoperoxide synthase 2 were upregulated under L-Ser-depleted conditions; this was suppressed by the addition of N-acetyl-L-cysteine. Thus, intracellular L-Ser deficiency triggers an inflammatory response via increased oxidative stress, and de novo L-Ser synthesis suppresses oxidative stress damage and inflammation when the external L-Ser supply is restricted.

Abbreviations
Atf4, activating transcription factor 4; EMEM, Eagle’s minimum essential medium; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione; ISR, integrated stress response; MEF, mouse embryonic fibroblast; Phgdh, 3-phosphoglycerate dehydrogenase; Ptgs2, prostaglandin-endoperoxide synthase 2; qRT-PCR, quantitative real-time PCR; l-Ser, l-serine; Txnip, thioredoxin-interacting protein.

L-Serine (l-Ser) is synthesized de novo from 3-phosphoglycerate via the phosphorylated pathway in which 3-phosphoglycerate dehydrogenase (Phgdh) catalyzes the first step reaction. l-Ser serves as a necessary precursor for the synthesis of proteins, sphingolipids, glycerophospholipids, folic acid metabolites, and amino acids such as glycine (Gly) and l-cysteine (l-Cys). Furthermore, the conversion of l-Ser into Gly participates in the biosynthesis of purines and pyrimidines, by transferring a one-carbon unit to tetrahydrofolate (THF). Our previous in vivo study demonstrated that severe l-Ser deficiency in mice with systemic targeted disruption of Phgdh resulted in intrauterine growth retardation, multiple organ malformation, and
embryonic lethality [1,2]. L-Ser biosynthesis defects in humans resulting from Phgdh mutations were identified to be Ser synthesis disorders and Neu–Laxova syndrome, the symptoms of which are characterized by severe fetal growth retardation, microcephaly, and perinatal lethality [3–5]. These findings have demonstrated that de novo L-Ser synthesis is essential for embryonic development and survival in mice and humans.

We recently reported that reduced availability of intracellular L-Ser promotes the biosynthesis and accumulation of 1-deoxysphinganine (doxSA) and its metabolites 1-deoxysphingolipids in mouse embryonic fibroblasts (MEFs) lacking functional Phgdh (KO-MEFs) [6]. The condensation of palmitoyl-CoA with L-Ala instead of L-Ser generated doxSA and its biosynthesis defects in KO-MEFs even in the presence of L-Ser [7]. Consistent with these observations, our microarray analysis of L-Ser-depleted KO-MEFs revealed that the activation of a network containing the stress-response-activating transcription factor ATF4–ATF3–DNA damage-inducible transcript 3 (Ddit3) axis was most prominent among the 560 upregulated genes [8], implying that L-Ser deficiency causes metabolic stress in KO-MEFs. However, the causal link between reduced L-Ser availability and vulnerability to stress remains unexplored.

Here, we show that L-Ser-depleted KO-MEFs are vulnerable to oxidative stress, which is accompanied by increased expression of thioredoxin-interacting protein (Txnip), a mediator of oxidative stress to inflammation, and the proinflammatory enzyme prostaglandin-endoperoxide synthase 2 (Ptgs2; also known as cyclooxygenase (COX) 2). These findings suggest that L-Ser deficiency leads to an inflammatory response through diminished protection against oxidative stress.

**Materials and methods**

**Cell culture**

Frozen stocks of immortalized wild-type (WT)- and Phgdh-knockout (KO)-MEFs were thawed and maintained in the complete medium, high-glucose Dulbecco’s modified Eagle’s medium (DMEM; Wako Pure Chemical Industries, Osaka, Japan), containing 10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 10 µg·mL⁻¹ gentamicin (Nacalai Tesque, Kyoto, Japan) in a humidified atmosphere at 37 °C with 5% CO₂ [2]. To deprive MEFs of L-Ser, the complete medium was replaced with Eagle’s minimum essential medium (EMEM; Wako Pure Chemical Industries) supplemented with 1% FBS and 10 µg·mL⁻¹ gentamicin, which contained all essential amino acids and l-glutamine but did not include L-Ala, L-Asp, L-Asn, L-Cys, L-Glu, Gly, L-Pro, and L-Ser. This medium is defined as the L-Ser-depleted condition, which contained 4 µM L-Ser derived from FBS. The L-Ser-supplemented condition was established by adding l-Ser (final 400 µM) to EMEM supplemented with 1% FBS and 10 µg·mL⁻¹ gentamicin. In some experiments, KO-MEF lines were retrovirally transduced with mouse Phgdh cDNA (KO-MEF⁺ Phgdh) or green fluorescent protein cDNA (Gfp; KO-MEF⁺ Gfp) [2] and with Atf4 short hairpin RNA (shAtf4) as previously described (T. Sayano, Y. Kawano, K. Takashima, W. Kusada, M. Udone, Y. Katakura, T. Ogawa, Y. Hirabayashi, S. Furuya, manuscript in preparation); total RNA was extracted after 6 h incubation for quantitative real-time PCR (qRT-PCR). To deprive MEFs of L-Leu, the medium was replaced with DMEM (deficient in L-Leu, L-Arg, and L-Lys, low glucose; Sigma-Aldrich Japan; Tokyo, Japan) containing 1% FBS, gentamicin, 800 µM l-Lys, and 400 µM l-Arg, with or without 800 µM l-Leu.

**Total glutathione quantification**

Knockout-MEFs grown in DMEM with 10% FBS and 10 µg·mL⁻¹ gentamicin were replated in either l-Ser-supplemented (at 40% cell confluence) or L-Ser-depleted (at 80% cell confluence) conditions for 24 h. Cells maintained under both conditions reached 80% cell confluence and were washed with Dulbecco’s phosphate-buffered saline (DPBS) followed by scraping from the dishes with DPBS. The cell suspensions were centrifuged at 1500 g, and each pellet was resuspended and lysed with 80 µL of 10 mM HCl. The lysates were alternately frozen and thawed twice, after which 20 µL of 5% (w/v) 5-sulfosalicylic acid was added. The lysates were centrifuged at 8000 g for 10 min, and the supernatants were used for glutathione (GSH) measurement. The total GSH levels were quantified using the GSSG/GSH Quantification kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s protocol, on a Multiskan™ FC microplate photometer (Thermo Fisher Scientific).

**Measurement of intracellular H₂O₂ generation**

Knockout-MEFs were seeded at 5 × 10³ to 1 × 10⁴ cells per well on Clear Fluorescence Black Plates (Greiner Bio-One International GmbH, Frickenhausen, Germany) in 100 µL of complete medium and incubated overnight at 37 °C, after which the medium was replaced with EMEM containing 1% FBS with or without l-Ser and incubated for 6 h. To detect endogenous H₂O₂ within cells, KO-MEFs were washed with DPBS and incubated with 2 mM BES-H₂O₂-Ac, a cell-permeable fluorescent probe for H₂O₂ (Wako...
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Pure Chemical Industries) [9], and Hoechst 33342 (Dojindo Laboratories) for 20 min. Images were acquired using the In Cell Analyzer 1000 (GE Healthcare UK Ltd., Buckinghamshire, UK) using 360- and 492-nm excitation filters, and 460- and 535-nm emission filters, as previously described [10]. The threshold of BES-H\textsubscript{2}O\textsubscript{2}-Ac intensity was set to the point at which approximately 75% of l-Ser-supplemented KO-MEFs were negative, and cells were scored as positive or negative using SPOTFIRE DECISIONSITE CLIENT 8.2 software (GE Healthcare Japan, Tokyo, Japan). This software was used to visualize and analyze the results [11,12].

**Cell viability assay**

Wild-type- and KO-MEFs were seeded at 4 × 10\textsuperscript{5}–1 × 10\textsuperscript{6} cells per well in 96-well plates in 100 μL of the complete medium and incubated overnight (12–24 h) at 37 °C. The medium was changed to EMEM containing 10% FBS and H\textsubscript{2}O\textsubscript{2} (0.01, 0.1, 1, 5, or 10 μM), and cells were incubated for 6 h. Live cells were counted using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Cell Counting Kit-7; Dojindo Laboratories), which was added to each well and incubated at 37 °C for 1 h. After gentle shaking, the absorbance of the culture medium was measured at 450 nm.

**Isolation of total RNA and qRT-PCR**

Total RNA was extracted from MEFs using an RNA Isolation Kit (Roche Diagnostics Japan, Tokyo, Japan), and 1 μg of total RNA was used for cDNA synthesis. A High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies Japan Ltd.) was used as previously described [2], and qRT-PCR was performed with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) using THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan). The primers used were as follows: Tnnip forward, 5'-AGCAGGACATGGAAGCAAGTT-3', and reverse, 5'-TTCTTTTTCCAGCGGAGGAGA-3'; Ptgs2 forward, 5'-ACAGACTGTGCCACATCTCAAGC-3', and reverse, 5'-GATACTGGAACTGCTGGTTGAAAAG-3'; glyceraldehyde 3-phosphate dehydrogenase (Gapdh) forward, 5'-ACTCCCCACTCTCCACCTTCG-3', and reverse, 5'-ATGTAGCCATGAGGTCCACC-3'.

**Western blot analysis**

Cells were lysed to extract the total protein, which was fractionated using SDS/PAGE, and transferred to polyvinylidene difluoride membranes as previously described [2]. Membranes were probed with the primary antibodies: anti-Txnip (1 : 100 dilution; Medical & Biological Laboratories Company, Nagoya, Japan), anti-Cox2 (1 : 500 dilution; Cell Signaling Technology Japan K.K., Tokyo, Japan), and anti-Gapdh (1 : 100 000 dilution; EMD Millipore, Billerica, MA, USA). Bound antibodies were visualized and quantified as previously described [2].

**Antioxidant treatments**

Knockout-MEFs were cultured in complete medium for 20 h, after which the culture medium was changed to EMEM containing 1% FBS in the absence of l-Ser. N-acetyl-l-cysteine (NAC) was added to the culture medium at concentrations of 1 or 5 mM for 6 h, after which total RNA was extracted and used for qRT-PCR as described above.

**Statistical analyses**

Data were evaluated using t-tests to analyze differences between two groups. To analyze differences among more than two groups, one-way analysis of variance followed by Dunnett’s post hoc test was used. P-values < 0.05 were considered significant. Data are expressed as the means ± standard error. All statistical analyses were performed using KALEIDAGRAPH 4.0 (Synergy Software, Reading, PA, USA).

**Results**

l-Ser deficiency reduced glutathione and increased vulnerability to oxidative stress in Phgdh KO-MEFs

We have previously reported that l-Ser depletion reduces the intracellular levels of Gly, Cys, and l-Ser [2]. As both Gly and Cys are necessary precursors of GSH, we compared the total GSH levels in l-Ser-supplemented and l-Ser-depleted KO-MEFs. Figure 1A shows that intracellular GSH levels were reduced significantly in KO-MEFs under l-Ser-depleted conditions compared to l-Ser-supplemented conditions. We then sought to determine whether endogenous production of intracellular H\textsubscript{2}O\textsubscript{2} was altered in KO-MEFs under l-Ser-depleted conditions using BES-H\textsubscript{2}O\textsubscript{2}-Ac, a cell-permeable fluorescent dye for H\textsubscript{2}O\textsubscript{2} [9]. After 6-h incubation, the percentage of BES-H\textsubscript{2}O\textsubscript{2} positive cells in l-Ser-depleted KO-MEFs was significantly increased compared to the l-Ser-supplemented condition (Fig. 1B). Next, we examined the effect of H\textsubscript{2}O\textsubscript{2} treatment on cell viability and observed that increasing the concentration of H\textsubscript{2}O\textsubscript{2} reduced the viability of both types of MEFs (Fig. 1C). However, KO-MEFs were more vulnerable to H\textsubscript{2}O\textsubscript{2} than WT-MEFs at lower H\textsubscript{2}O\textsubscript{2} concentrations (0.1 and 0.01 μM). These observations indicated that the loss of de novo l-Ser synthesis culminated in enhanced H\textsubscript{2}O\textsubscript{2} generation and vulnerability to its oxidative stress.
L-Ser depletion upregulates Txnip and Ptgs2 expression in KO-MEFs

To verify whether L-Ser deficiency affects the oxidative stress response in KO-MEFs, we focused on Txnip, a multifunctional protein linking oxidative stress to inflammation [12]. Txnip was identified by microarray analysis as being upregulated in L-Ser-depleted KO-MEFs [7,8] and transcriptionally activated by increased H2O2 [13]. First, we compared Txnip mRNA levels in KO- and WT-MEFs under L-Ser-supplemented or L-Ser-depleted conditions. After incubation in L-Ser-depleted medium for 6 h, an 8-fold increase in Txnip mRNA was detected in KO-MEFs but not in WT-MEFs (Fig. 2A). Consistently, Txnip protein expression level in L-Ser-depleted KO-MEFs significantly increased to 1.8-fold higher than that in L-Ser-supplemented KO-MEFs (Fig. 2B). To examine whether Txnip mRNA induction was due to Phgdh deletion, we measured the Txnip mRNA levels in KO-MEFs + Phgdh and in KO-MEFs + Gfp under L-Ser-depleted conditions. Viral transduction of Phgdh, but not Gfp, suppressed Txnip mRNA induction under L-Ser-depleted conditions (Fig. 2C), indicating that loss of Phgdh was primarily responsible for Txnip induction under L-Ser-depleted conditions. Time course analysis of Txnip mRNA expression demonstrated a sharp 7-fold increase as early as 2 h after the exposure of L-Ser-depleted medium to KO-MEFs, and a significant 3-fold increase 24 h after exposure (Fig. 2D).

Next, we tested whether the expression of Ptgs2, a proinflammatory enzyme, was also induced in L-Ser-depleted KO-MEFs, because Txnip participates in the regulation of Ptgs2 expression [14,15]. We compared Ptgs2 mRNA levels in KO-MEFs and found that after 6-h incubation, a substantial 16-fold increase in Ptgs2 mRNA was detected in the L-Ser-depleted condition compared to the L-Ser-supplemented condition, while WT-MEFs did not show such an increase in L-Ser-depleted conditions (Fig. 2E). Accordingly, a significant increase in Ptgs2 protein was observed in L-Ser-depleted KO-MEFs compared to those in L-Ser-supplemented conditions (Fig. 2F). As with Txnip (Fig. 2C), viral transduction of Phgdh cDNA but not Gfp suppressed Ptgs2 mRNA induction (Fig. 2G). Time course analysis of Ptgs2 mRNA expression demonstrated a subtle but significant 1.3-fold increase after 2-h incubation of KO-MEFs in L-Ser-depleted medium, which reached a plateau after 6-h incubation, and retained a 1.4-fold increase even after 24 h (Fig. 2H). These observations indicated that reduced L-Ser availability caused by Phgdh disruption results in the upregulation of both mRNA and protein levels of Txnip and Ptgs2 within 6 h.

Transcriptional activation of Txnip and Ptgs2 is independent of the integrated stress response pathway

To gain insight into the underlying molecular mechanisms by which Txnip expression was upregulated under L-Ser-depleted conditions, we examined whether the integrated stress response (ISR) pathway, which is
activated by amino acid deficiency, regulated Txnip expression in L-Ser-depleted KO-MEFs. It has been well documented that deprivation of one or more amino acids can induce the activation of the ISR pathway, which results in enhanced phosphorylation of the translation initiation factor 2α and subsequent
increased expression of the transcription factor Atf4 [16,17]. As a result of amino acid deprivation, Atf4 target genes are substantially induced [18]. As we have demonstrated that L-Ser depletion in KO-MEFs causes a robust increase in Atf4 protein expression (Sayano et al., manuscript in preparation) and upregulation of several Atf4-target genes [2,7], we sought to determine whether Tnxip and Ptgs2 induction was regulated by Atf4 via the ISR pathway. To evaluate the functional involvement of Atf4 in KO-MEF transcription, we generated shRNA-mediated Atf4 knockdown (KD)-KO-MEFs, in which Atf4 protein expression was suppressed by 0.4-fold under L-Ser-depleted conditions compared to mock-treated KO-MEFs (Sayano et al., manuscript in preparation). As shown in Fig. 3A,B, Tnxip and Ptgs2 induction in Atf4 KD-KO-MEFs was unchanged compared to mock KO-MEFs under L-Ser-depleted conditions, suggesting that Atf4 and the ISR pathway did not play a role in Tnxip and Ptgs2 induction in L-Ser-depleted KO-MEFs. The ISR pathway is activated in response to the deficiency of indispensable amino acids, especially L-Leu [16–18].

We then investigated whether the depletion of the indispensable amino acid L-Leu could induce Tnxip and Ptgs2 expression in KO-MEFs. Figure 3C,D shows that L-Leu depletion elicited significant increases in Tnxip and Ptgs2 expression, although the magnitudes of induction were lower than those observed during L-Ser-depleted conditions (Fig. 3C,D). These observations suggest that L-Ser deficiency plays a more profound role in Tnxip and Ptgs2 expression than L-Leu deficiency.

Antioxidant addition suppresses Tnxip and Ptgs2 expression caused by L-Ser depletion

To clarify the upstream mechanism underlying the upregulation of Tnxip and Ptgs2 seen in L-Ser-deficient KO-MEFs, we examined the effects of antioxidant treatment on mRNA expression. The addition of NAC, a GSH precursor, caused significant suppression of mRNA expression of both Tnxip and Ptgs2 in KO-MEFs under L-Ser-depleted conditions (Fig. 4A,B). These results suggest that increasing oxidative stress elicited by L-Ser depletion causes the upregulation of Tnxip and Ptgs2 in KO-MEFs.

Discussion

This study demonstrated that intracellular L-Ser deficiency caused by Phgdh deletion and external L-Ser depletion elicited increased vulnerability to oxidative stress by increasing the expression of Tnxip and Ptgs2. The results also showed that antioxidant treatment, such as the addition of NAC, suppressed the induction of Tnxip and Ptgs2 under L-Ser-depleted conditions.

Fig. 3. Tnxip and Ptgs2 induction is not associated with the ISR pathway activated by amino acid deficiency in L-Ser-depleted KO-MEFs. (A,B) Mock- and shAtf4-transduced KO-MEFs were cultured under L-Ser-supplemented or L-Ser-depleted conditions for 6 h, and Tnxip (A) and Ptgs2 (B) mRNA levels were measured (mock-transduced KO-MEFs, n = 3; shAtf4-transduced KO-MEFs, n = 3; Student’s t-test, *P < 0.05, **P < 0.005). (C,D) KO-MEFs were cultured under L-Leu-supplemented or L-Leu-depleted conditions for 6 h, and Tnxip (C) and Ptgs2 (D) mRNA levels were measured (KO-MEFs, n = 3, Student’s t-test, *P < 0.05, **P < 0.005, ***P < 0.0005).
stress via the reduction in GSH, which led to the induction of \( \text{Txnip} \) and \( \text{Ptgs2} \) expression in nonmalignant MEFs. GSH is synthesized from \( \text{L-Glu} \), \( \text{L-Cys} \), and \( \text{Gly} \) and prevents damage to cellular components against oxidative stress generated by intracellular reactive oxygen species (ROS). Several studies in cancer cells have reported that the intracellular levels of \( \text{L-Ser} \), \( \text{Gly} \), and GSH decrease after exposure to \( \text{H}_2\text{O}_2 \) [19], and \( \text{L-Ser} \) and \( \text{Gly} \) depletion leads to decreased GSH levels and increased cell death in \( \text{p53}^{-/-} \) or \( \text{p21}^{-/-} \) HCT116 cells [20]. Our observations in KO-MEFs, indicating that the \( \text{Phgdh} \)-dependent \( \text{L-Ser} \) biosynthetic pathway plays a primary role for maintaining the intracellular GSH level and preventing cell death under nutritional stress conditions, are consistent with these reports [21,22].

We demonstrated that \( \text{L-Ser} \) depletion induced \( \text{Txnip} \) expression in KO-MEFs. \( \text{Txnip} \), also known as vitamin D3-upregulated protein 1, was originally identified as a negative regulator of thioredoxin 1/2 (Trx), a key sensor of cellular redox status that regulates protection against oxidative stress. The Trx–\( \text{Txnip} \) complex is a critical regulator of intra- and extracellular redox signaling and ROS [23]. \( \text{Txnip} \) expression is upregulated by ROS [12,24,25] and oxidative stress caused by ischemic–reperfusion injuries [26,27]. This study showed that \( \text{Txnip} \) expression was induced by \( \text{L-Ser} \) depletion in KO-MEFs, and this was inhibited by viral transduction of \( \text{Phgdh} \) cDNA (Fig. 2A–C). It is well documented that \( \text{Txnip} \) links oxidative stress to inflammation by activating NLR family pyrin domain containing 3 [11,28,29], and participates in the upregulation of \( \text{Ptgs2} \) expression [13,14]. The ISR pathway is an oxidative stress defense mechanism in response to essential amino acid deficiency [16]. This study showed that \( \text{Txnip} \) induction caused by \( \text{L-Ser} \) deficiency was not suppressed by \( \text{Atf4} \) KD in \( \text{L-Ser} \)-depleted KO-MEFs (Fig. 3A), indicating that \( \text{Txnip} \) induction is independent of the ISR pathway. \( \text{Txnip} \) induction by \( \text{L-Ser} \) depletion was significantly suppressed by the addition of an antioxidant (Fig. 4A). These observations suggest that \( \text{Txnip} \) expression is increased by reduced \( \text{L-Ser} \) availability, linking aberrant redox regulation and the induction of an inflammatory response to \( \text{L-Ser} \) deficiency that is independent of the ISR pathway.

\( \text{Txnip} \) affects the inflammatory response and cell death signaling by regulating the cellular redox status [30], and loss of \( \text{Txnip} \) can lead to the proliferation of cancer cells [31]. We previously reported that \( \text{L-Ser} \)-depleted KO-MEFs exhibited cell growth arrest and increased cell death after 96-h incubation, which was associated with diminished mRNA translation and aberrant sphingolipid metabolism [2,6,7]. In addition to GSH, proteins, and lipids, \( \text{Phgdh} \) expression has been proved to be critical to maintain molecules important for cell proliferation, including reduced nicotinamide adenine dinucleotide phosphate [32], purine nucleotides [33], and THF metabolites [18,33]. Taken together, the present study implies that the loss of \( \text{de novo} \) \( \text{L-Ser} \) biosynthesis leads to cell proliferation arrest, followed by oxidative stress and inflammation, which seems to be a more severe cellular consequence compared to the loss of other nonessential amino acids but comparable to the severe phenotypes caused by the genetic deficiency of \( \text{Gln} \) [34]. We demonstrated that \( \text{L-Ser} \) deficiency promotes the biosynthesis and accumulation of doxSA, which can activate p38 MAPK in \( \text{L-Ser} \)-depleted KO-MEFs [7]. As \( \text{Txnip} \) expression was induced by \( \text{H}_2\text{O}_2 \) via p38 MAPK activation in human aortic smooth muscle cells [35], further study is needed to clarify whether p38 MAPK regulates \( \text{Txnip} \) induction in \( \text{L-Ser} \)-depleted KO-MEFs. These insights might contribute to the elucidation of the pathobiology of patients with Neu–Laxova syndrome/\( \text{L-Ser} \) deficiency disorders [3–5,34] or diseases associated with increased \( \text{Txnip} \) expression such as diabetes and obesity [23].
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Author contributions
MH and SF designed the study and wrote the manuscript. MH, YH, TS, YA, CZ, YK, and KM performed the experiments. TS, MU, and YK supervised contributed to the analysis of microarray data.

Conflict of interest
The authors declare no conflict of interest.

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