Hypoxia induces chemoresistance to proteasome inhibitors through orchestrating deSUMOylation and ubiquitination of SRC-3 in multiple myeloma

Jing Guo1,7, Yangyang Lv1,7, Sheng Wang3, Ziyi Peng2, Ying Xie2, Yixuan Wang2, Hongmei Jiang2, Xin Li2, Mengqi Wang2, Meilin Hu3, Jianguo Mu4, Jingya Wang2, Yangyang Xie2, Xiankui Cheng5, Zhigang Zhao1,6,8 and Zhiqiang Liu1,2,8

The bone marrow microenvironment in multiple myeloma (MM) is hypoxic and provides multi-advantages for the initiation of chemoresistance, but the underlying mechanisms and key regulators are still indistinct. In the current study, we found that hypoxia stimulus easily induced chemoresistance to proteasome inhibitors (PIs), and the steroid receptor coactivator 3 (SRC-3) expression was remarkably augmented at posttranslational level. Protein interactome analysis identified SENP1 as a key modifier of SRC-3 stability, as SENP1-mediated deSUMOylation attenuated the K11-linked polyubiquitination of SRC-3. SENP1 depletion in the MM cells showed impaired SRC3 stability, and knockdown of SENP1 in MM cells by CRISPR/cas9 sgRNA accelerated the degradation of SRC-3 and remarkably overcame the resistance to PIs. In the Vk*Myc and STG111 mouse models as well as patient-derived xenograft (PDX) of myeloma, SENP1 inhibitor Momordin Ic (Mc) increased the sensitivity to PIs in MM cells. Importantly, SENP1 level was positively correlated with SRC-3 level in the tissues from refractory/relapsed MM, as well as in xenograft tissues from mice treated with bortezomib and Mc. Taken together, our findings suggest that hypoxia-induced SENP1 is a crucial regulator of chemoresistance to PIs, and shed light on developing therapeutic strategies to overcome chemoresistance by using small molecules targeting SENP1 or SRC-3.

INTRODUCTION
Multiple myeloma (MM) is a hematologic malignancy of plasma cells accumulating mainly in the bone marrow, and results in secretion of excessive parafunctional monoclonal immunoglobulin protein and end-organ damage [1]. Investigations in molecular mechanisms on bench and the successful application of proteasome inhibitors on bench have led to the significant overall survival of MM patients [2, 3]. However, MM remains incurable and fatal, which is mainly caused by the occurrence of drug-resistant subclones during therapy [4]. A better understanding of the mechanisms of drug resistance in MM cells is urgently required.

MM cells originate and reside in the bone marrow (BM) [5]. It has been reported that the BM niche confers survival and chemoresistance of MM cells by a complex interplay of cytokines, chemokines, adhesion molecules, proteolytic enzymes, and other components of the extracellular matrix [6–8]. Besides, BM has long been regarded as a naturally hypoxic organ [9, 10]. A study using the ST33 MM murine model found that the myelomatous BM was more hypoxic than the normal bone marrow [11]. During MM progression disease, hypoxia BM niche exert strong selective pressure that shapes tumor evolution, make them adapt to reduced oxygen availability, become highly aggressive and resistant to treatment [12]. For instance, our previous study revealed that hypoxia promotes disease progression and bone lesion through upregulating DKK1 expression [13]. Given that hypoxia promotes tumor progression in solid malignancies, for example, live and breast cancers [14, 15], it is likely that MM are strongly influenced by hypoxia. However, the role of hypoxia in MM drug resistance is still elusive, and it is pivotal to clarify the mechanisms by which hypoxia promotes the pathogenesis and chemoresistance of MM.

Highly dynamic post-translational modifications (PTMs), including acetylation, phosphorylation, methylation, ubiquitination, SUMOylation, and NEDDylation, control accumulation and functions of proteins and are pivotal for carcinogenesis and disease progression [16, 17]. Actually, post-translational...
modifications such as ubiquitination and SUMOylation play even critical roles in MM than other solid tumors, since the most effective drugs for management of MM in clinic, such as proteasome inhibitors (PIs) and immunomodulatory drugs (iMiDs), disturb the ubiquitin-proteasome system, thus control proteasome inhibitors (PIs) and immunomodulatory drugs most effective drugs for management of MM in clinic, such as Bortezomib (BTZ) for 48 h. In this study, we investigated the effect of hypoxia on chemosensitivity to protease inhibitor of MM cells.

RESULTS

Hypoxia induces Bortezomib resistance in MM cells

Hypoxia has been known as a critical factor for drug resistance in ample solid tumors, therefore we investigated whether hypoxia influences the sensitivities of MM cells to effective drugs in clinic, such as Bortezomib (BTZ), carfilzomib (CFZ), and melphalan (Mel). The sensitivity to BTZ of two MM cells, MM.1S and LP-1, were all obviously receded under hypoxia stimulus compared to the normoxia control (Fig. 1A, B), as also shown by the significantly augmented half-maximal inhibitory concentration (IC50) values (Fig. 1C), the distinct abrogated cleavage of PARP as a marker of cell apoptosis (Fig. 1D), and the remarkable suppressed apoptotic cell rates (Fig. 1E). Intriguingly, hypoxia yielded similar chemoresistance alteration when MM were treated with another proteasome inhibitor Carfilzomib (Fig. 1F–J), but failed to induce conspicuous consequences of Melphalan in MM cells (Fig. 1K–O). Thus, these results indicate that hypoxia more easily induces chemoresistance to protease inhibitor of MM cells.

Hypoxia upregulates SRC-3 level in MM cells

Our previous study has demonstrated high SRC-3 is associated with poor prognosis in MM, and overexpression of SRC-3 promotes BTZ resistance [19]. To dissert the correlation between SRC-3 and hypoxia, we assessed whether hypoxia affects SRC-3 expression in MM cells at mRNA or protein levels. We confirmed that hypoxia induced the augmentation at SRC-3 protein level in a time-dependent manner, and hypoxia obviously prolonged the half-life of SRC-3 protein in MM cells (Fig. 2A), but the elevation of SRC-3 protein did not coordinate with the alteration trend of HIF-1α, nor at mRNA level (Fig. 2A, B). Immunofluorescence assay
We further used co-immunoprecipitation to confirm the physical interaction of SRC-3 with ubiquitin, and identified that SRC-3 was degraded mainly through lysine 11 (K11)-linked polyubiquitination, partially through K6-, and K29-linked polyubiquitination (Fig. 3C). However, when the K6−, K11−, and K29− lysine positive mutations of ubiquitin were overexpressed in MM cells together with SRC-3, we found that SRC-3 was modified through K11-linked polyubiquitination, since only dose-dependent modification of K11-linked polyubiquitination was elicited (Fig. 3D, E) on SRC-3, we failed to observe the K6- and K29-linked polyubiquitination (Fig. 3F, G). Moreover, when the K11 lysine was mutate to arginine, polyubiquitination of SRC-3 could barely be detected (Fig. 3H). Thus, these results indicate that SRC-3 protein is mainly modified through K11-linked polyubiquitination for degradation by the 26S proteasome.

**Senp1 stabilizes SRC-3 through deSUMOylation**

Next, we sought to deduce the key regulators for SRC-3 protein degradation. We dissected the interactome of SRC-3 in MM.1S cells under hypoxia stimulation using mass spectrometry. Intriguingly, we did not find any E3 ligase in the components of SRC-3 interactome, on the contrary, we discovered a cysteine protease of sentrin-specific protease (SENP) family, SENP1, which reverses SUMO conjugation on target proteins (Fig. 4A). The interaction between SRC-3 and SENP1 was bilaterally confirmed by co-immunoprecipitation in the HEK293T cells, either using HA-SNP1or Flag-SRC-3 as bait antibodies (Fig. S 2A). Moreover, the endogenous interaction of SRC-3 and SENP1 was also validated in MM cells using anti-SRC-3 or anti-SENP1 antibodies, respectively (Fig. 4B). In addition, protein level of SRC-3 was positively related to the exogenously expressed SENP1 in MM cells (Fig. 4C). These data suggested that degradation of SRC-3 might be regulated through coordination of SUMOylation and ubiquitination.

Previous studies have shown that transactivation activity of SRC-3 could be regulated by SUMOylation [21]. Actually, convergence of ubiquitination and SUMOylation in modulation of SRC-3 activity indicates the biological importance of their crosstalk. 

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Fig. 2 Hypoxia upregulates SRC-3 level in MM cells. A Western Blotting and immunofluorescence to test the levels of SRC-3, HIF-1α in LP-1 and MM.1S cells under hypoxia and normoxia in indicated time. Scale bar, 10 µm. B qPCR shows the expression of NCOA3 in LP-1 and MM.1S cells—under hypoxia condition with indicated time. C Western Blotting shows β-actin, SRC-3, HIF-1α expressions in wild type (WT) and bortezomb (BTZ)-resistant (BR) MM cells. D qPCR shows the expression of NCOA3 in WT and BR-MM cells. E, F Alteration of IC50 to BTZ treatment under hypoxia condition in the presence or absence of 25 nM SI-2 for 48 hr in LP-1 and MM.1S cells. G Combination index plot obtained from the Compusyn Report for BTZ and SI-2 combinations. H Flow cytometry assay for apoptosis of LP-1 and MM.1S cells induced by 5 nM BTZ in the presence or absence of 25 nM SI-2 for 48 hr. I Cleavage of PARP as the apoptotic marker in MM cells under hypoxia condition in the presence or absence of 25 nM SI-2 for 48 hr, treated with increasing dosage of BTZ for 48 h. *P < 0.05, **P < 0.01, ***P < 0.001, two-sided P values were determined by Student's t-test for n = 3 biologically independent experiments.
of protein functions has emerged as a crucial cellular mechanism in regulating pathogenesis [25]. Our result showed that SRC-3 physically interacted with small ubiquitin-related modifier (SUMO) 1, SUMO2, and SUMO3, but the interaction with SUMO1 was dominant (Fig. S 2C). When SUMO1 was gradually overexpressed in MM cells, SRC-3 degradation was enhanced in a dose-dependent manner (Fig. S 2D); on the contrary, SUMO1 depletion resulted in attenuation of the SRC-3 ubiquitination both in HEK293T cells (Fig. S 2E) and in MM cells (Fig. S 2F, G). Thus, we confirmed that SUMOylation was involved in SRC-3 protein degradation. To further investigate the role of SUMOylation in SRC-3 stability in vivo, we generated Sumo1 knockout mouse, and confirmed that SUMO1 has been completely depleted in B cells (Fig. S 2H). In addition, the half-life of SRC-3 protein was markedly increased in the SUMO1−/− B cells (Fig. S 2I), and ubiquitination of SRC-3 was barely detected in the SUMO1−/− B cells (Fig. 4H).

Taken together, these results suggest that SUMOylation is involved in proteasome-dependent degradation of SRC-3. To further explore the role of SENP1 in SRC-3 modification, we knockdown SENP1 in the HEK293T cells that have stably expressed SUMO1 and flag-tagged SRC-3, and observed that SUMOylation of SRC-3 was markedly attenuated (Fig. 3A); on the contrary, overexpression of SENP1 (SENP1-OE) remarkably attenuated the ubiquitination modification of SRC-3 compared with the vector controls (Fig. 3B). Importantly, overexpression of SENP1 dramatically relieved the K11-linked ubiquitination, but not K6- and K29-linked ubiquitination of SRC-3 (Fig. 3C). We further suppressed SENP1 expression using lentivirus carrying shRNA in MM cells, and failed to observe significant attenuation on SRC-3 ubiquitination and SUMOylation, and simultaneously total SRC-3 protein level was unable to elevate under hypoxia stimulation (Fig. 4E, F). To confirm the phenotype of SENP1 in regulating SRC-3 stability in vivo, we generated a SENP1 conditional knockout mouse using SENP1-floxp and CD19-Cre crossed mice, and efficiently deleted SENP1 in the B cells (Fig. S 3C). As a consequence, protein level of SRC-3 in the SENP1−/− CD19-Cre B cells was obviously downregulated, and administration of MG132 failed to restore the protein level of SRC-3 compared with the B cells from wild type control mice (Fig. S 3D). Moreover, the half-life of SRC-3 protein was markedly shortened in the SENP1−/− CD19-Cre B cells compared with that in the wild type B cells (Fig. 4H). As expected, endogenous ubiquitination and SUMOylation of SRC-3 was more readily detected in the SENP1−/− CD19-Cre B cells but not in wild type B cells (Fig. 4H). Collectively, our results indicate that SENP1 protects SRC-3 against degradation by SUMOylation-associated ubiquitination.

SENP1 is a downstream target of HIF-1α in MM cells

We next assessed expressions of the SENP family genes with HIF-1α overexpression (HIF-1α OE) in MM cells, and identified that hypoxia dominantly induced the expression of SENP1 in protein and mRNA level (Fig. 5A, B), the results were similar with that under hypoxia condition in MM cells (Fig. 5C, D). On the contrary, when HIF-1α was knocked down by lentivirus carrying shRNAs in MM cells (Fig. 5E), SENP1 expression was dramatically suppressed both at mRNA and at protein levels, together with the downregulated of SRC-3 only at protein level (Fig. 5F). Interestingly, we verified the similar expression pattern of SENP1 in the BR-MM cells (Fig. 5G, H). To determine whether HIF-1α is a transcriptional factor for SENP1, we constructed a SENP1-luciferase reporter containing two cis-acting elements of HIF-1α at −102 to −111bp and −1684 to −1691bp (Fig. 5I), and ectopically expression of HIF-1α resulted in over 10 folds activation of the SENP1-luciferase reporter (Fig. 5J). Importantly, hypoxia triggered significant enrichment of HIF-1α on SENP1 promoter in MM cells when detected by chromatin

Fig. 3 Degradation of SRC-3 is ubiquitin-proteasome dependent. A Levels of SRC-3 in LP-1 and MM.1 S cells treated with increasing dosage of BTZ for 24 h. B Co-immunoprecipitation (Co-IP) assay shows interactions between Ubiquitination and SRC-3. Input, 2% lysate. IP, M2-flag antibody. C Co-IP assay shows the interactions between Ub-WT, Ub-K6, Ub-K11, Ub-K27, Ub-K29, Ub-K33, Ub-K48, Ub-K63 and SRC-3. Input, 2% lysate. IP, M2-flag antibody. D SRC-3-3 × flag levels in HEK293T cells co-transfected with Ub-WT for 48 h and treated with MG132 (10 mM) for 4 h before harvesting proteins were detected by anti-flag antibody. E SRC-3-3 × flag levels in HEK293T cells co-transfected with Ub-K6 for 48 h and treated with MG132 (10 mM) for 4 h before harvesting proteins were detected by anti-flag antibody. F SRC-3-3 × flag levels in HEK293T cells co-transfected with Ub-K6 for 48 h and treated with MG132 (10 mM) for 4 h before harvesting proteins were detected by anti-flag antibody. G SRC-3-3 × flag levels in HEK293T cells co-transfected with Ub-K29 for 48 h and treated with MG132 (10 mM) for 4 h before harvesting proteins were detected by anti-flag antibody. H Co-IP assay shows interactions between SRC-3 and Ub-K11 or Ub-K11R. Input, 2% lysate. IP, M2-flag antibody.
**Fig. 4** SENP1 stabilize SRC-3 through deSUMOylation. A Silver staining of MM.1S cells infected with lentivirus carrying SRC-3-3×-flag for 72 hr and then under hypoxia stimulation for 12 h. B Interaction between endogenous SRC-3 and SENP1 in MM.1S and LP-1 cells was detected by using SRC-3 antibody or reversely SENP1 antibody for IP. C Degradation of SRC-3 in MM.1S and LP-1 cells treated with or without MG132 and with or without SENP1 overexpression. D Indicated plasmids were co-transfected in HEK 293T cells and treated with MG132 (10 mM) for 4 h before harvesting protein, immunoprecipitated with anti-flag antibody. Bound proteins were detected by anti-HA IB. E, F Western Blotting shows SENP1, SRC-3, Ub, Sumo expressions in NT control and SENP1 KD MM cells under hypoxia and normoxia in indicated time. G The half-life of SRC-3 protein was decreased in SENP1 KD, CD19-cre B cells. H SUMOylation level of SRC-3 accumulated in SENP1 KD, CD19-cre B cells after treated with MG132. SENP1 KD, CD19-cre, and SENP1 KD B cells were treated with MG132 (10 mM) for 4 h as indicated. SRC-3 was immunoprecipitated with anti-SRC-3 antibody from cell lysates. The precipitates were immunoblotted (IB) with anti-Ub, anti-SUMO1 antibodies. I Ubiquitination of SRC-3 accumulated in Control B cells after treated with MG132. SUMO1−/− and Control B cells were treated with MG132 (10 mM) for 4 h as indicated. SRC-3 was immunoprecipitated with anti-SRC-3 antibody from cell lysates. The precipitates were immunoblotted (IB) with anti-Ub antibodies.

The resistance to BTZ treatment compared with its paired vector control (Figs. 6J, S 4F). Clinically, we observed that protein levels of HIF-1α, SENP1 and SRC-3 were mutually correlated, and the expressions were dramatically augmented in the refractory/relapsed MM patients (Figs. 6K, S 4G–J), and combination of SENP1 inhibitor Mc with BTZ dramatically augmented cell apoptosis in primary CD138+ plasma cells that have been resistant to BTZ-based regimens (Fig. 6L). In conclusion, these data suggest that SENP1 plays a critical role in regulating chemosensitivity to PIs in MM cells.

**Targeting SENP1 suppresses hypoxia-induced SRC-3 and drug resistance in vivo**

To assess the effect of targeting SENP1 in overcoming drug resistance in vivo, we established BTZ-resistant MM cell derived xenograft models, RRMM patient CD138+ plasma cell-derived intra-bone growth MM model, as well as the Vk*Myc transgenic and transplant mouse models of MM. The SENP1 inhibitor Mc alone administration had no obvious inhibitory effects on tumor growth, nor did BTZ alone administration, however, the combination of BTZ and Mc considerably suppressed the tumor growth of BR-MM (Fig. 7A), prolonged the survival rate of mice (Fig. 7B). Importantly, more cell apoptosis (Fig. 7C) and decreased expression of SENP1 and SRC-3 were obviously observed in the combined treatment groups (Fig. 7D). Moreover, in unsorted bone marrow mononuclear cells of three relapsed MM patients derived xenograft model, tumor burdens were also noticeably extenuated in the combination treatment groups, as evidenced by significantly suppressed M-protein levels (Fig. 7E) and decreased
CD138 cells percentage in the bone marrow (Fig. 7F). Furthermore, in the successfully constructed Vk*Myc transplant mouse models of MM (Fig. S 5A–D), we further validated the synergistic anti-MM effect of combination of SENP1 inhibitor and BTZ, as shown by the conspicuously reduced M-protein level (Fig. 7G), and nearly disappeared plasma cells in the bone marrow (Fig. 7I). Taken together, these in vivo data strongly suggest that pharmacologically targeting SENP1 abrogates chemoresistance to PIs in MM cells.

**DISCUSSION**

In the current study, we identified an important role of hypoxia in regulating chemosensitivity to PIs of MM cells. Our study showed that hypoxia enhanced SENP1 expression through HIF-1α, and SENP1 deSUMOylates SRC-3 via K11-linked ubiquitination, consequently protects the SRC-3 from 26S proteasome dependent degradation and favors MM cells survival. Translationally, our pre-clinical data suggests that using small molecule targeting SENP1 could re-sensitize resistant MM cells to PIs, which may benefit the strategy development for refractory or relapsed MM patients.

Our previous findings has revealed that the histone methyltransferase NSD2 protects SRC-3 from degradation through forming liquid-liquid phase separation and renders resistance to PIs [19]. SRC-3 promotes numerous aspects of cancer, such as initiation, progression, and chemoresistance [26], and suppression of SRC-3 levels and/or activity are efficient enough to alter its transcriptome [27, 28]. Several studies have demonstrated that stimuli could induce multiple posttranslational modifications of SRC-3, including phosphorylation, ubiquitination, SUMOylation, acetylation, and methylation [26, 29]. In this study, we report that hypoxia is a new stimulus for SRC-3 expression without affect its transcriptional level. It has been reported that hypoxia triggered activation of NF-κB signaling pathway to enhance bortezomib resistance in MM cells [30, 31]. Interestingly, our current study also discover that hypoxia mainly cause chemoresistance towards proteasome inhibitors, but not to other drugs such as Melphalan, a DNA alkylating drug inhibiting DNA and RNA synthesis [32]. Therefore, our study indicates that hypoxia may affect key regulators for chemosensitivity that depends on the ubiquitin-proteasome system. Since SRC-3 is degraded in a proteasome-dependent manner, thus it is very easily affected by hypoxia.

After deciphering the interactome of SRC-3 in MM cells under hypoxia condition, we unexpectedly discovered a deSUMOylation enzyme SENP1, but no ubiquitination-related E3 ligase, interact with SRC-3. SENP1 belongs to SUMO-specific proteases (SENP)s, which have a dual function as processing enzymes for pre-SUMO and deconjugates of SUMO conjugates [33]. Overexpression of SENP1 positively correlated with adverse events of tumor such as tumor differentiation, lymph node metastasis, and recurrence [34, 35]. Similar to its roles in solid tumors, our study demonstrates that SENP1 is an important regulator for chemoresistance to PIs in MM cells under hypoxia condition. Small ubiquitin-like modifiers (SUMOs) are conservatively expressed in all eukaryotes, engender protein SUMOylation modification, and are essential for the maintenance of genomic integrity and the regulation of gene expression and intracellular signaling [36]. Our present study provides the first evidence that SUMOylation coordinates...
with ubiquitination in regulating the stability of SRC-3, providing a novel knowledge for understanding the regulation of SRC-3 stability in MM cells under hypoxia condition. In solid tumors, it has been well established that hypoxia has a negative effect on the efficacy of radio- and chemo-therapy, through affecting drug delivery, DNA damage repair, regulation of genes governing drug resistance, as well as cell death pathways \[37, 38\]. The bone marrow which has been invaded by MM cells contains a heterogeneous range of oxygen pressures due to rapidly proliferating cells and angiogenesis, but the average oxygen pressure generally is under the normoxia range. In this study, we provided evidences that under hypoxia, HIF-1α-SENP1 axis modulates SRC-3 stability, but hypoxia does not directly regulate SRC-3 transcription. Thus, we conclude that hypoxia promotes the transcription of SENP1 through HIF-1α, and protects SRC-3 protein from degradation by SUMOylation-mediated ubiquitination. Since bortezomib treatment leads to SRC-3 accumulation, and SRC-3 overexpression confers resistance to PIs, our study suggests a positive feedback leading to MM drug resistance.

In summary, this study provides new knowledge for understanding the chemoresistance of MM cells to PIs under hypoxia condition, and further emphasizes the importance of SRC-3 in regulating sensitivity to PIs via its interaction with the SUMO-specific protease SENP1. Our results also shed light on the development of therapeutic strategies to overcome refractory or relapse in MM patients using the SENP1 inhibitor in clinic.

MATERIALS AND METHODS

Ethics approval and consent to participate
This study was approved by the Ethics Committee of Tianjin Medical University, and all protocols conformed to the Ethical Guidelines of the World Medical Association Declaration of Helsinki. Signed informed consent was obtained from each participating individual prior to participation in the study. All animal studies performed during this experiment were approved by the Committee on Animal Research and Ethics of Tianjin Medical University, and all protocols followed the Guidelines of Ethical Conduct in the Care and Use of Nonhuman Animals in Research.

Patient-derived xenograft (PDX) mouse model for MM
To develop a PDX model, unsorted bone marrow mononuclear cells containing \(0.5\times10^5\) CD138\(^+\) plasma cells from MM patients with relapse after bortezomib-based treatments. PCs were cultured on bone marrow stromal cells and treated with bortezomib (5 nM) and Mc (25 µM) for 12 h. \(*P<0.05, **P<0.01, ***P<0.001\), two-sided \(P\) values were determined by Student’s \(t\)-test for \(n=3\) biologically independent experiments.
The Vk*Myc transgenic and transplant mouse models of MM
The Vk*Myc transgenic and transplant mouse model were established according to Dr. Bergsagel PL’s report [22]. Briefly, 0.5 × 10^6 cells/mouse were injected via tail vein of 5- to 12-week-old C57BL/6 wild-type recipient mice (n = 6 per group). One week after transplantation, mice were divided into 4 groups randomly and treated with Mc (10 mg/kg, third weekly, n = 6), BTZ (1 mg/kg, third weekly, n = 6), the combination of both drugs (n = 6) or vehicle control (DMSO, n = 6) for 4 weeks. Serum paraprotein was assessed on day 1 and then weekly for 5 weeks and presented as mean change from levels on day 0 (mean SEM).

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AUTHOR CONTRIBUTIONS

LG and ZQL contributed to writing the manuscript; JG, YLY, SW, YX, HM, XL, ZYP, YKK, and JPM contributed to performing the experiments and statistical analyses; JG, SW, MLH, and MQW were in charge of the animal studies; ZQL and XKC provided the patient samples and clinical statistics; ZQL and ZGZ contributed to the final version of the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

CONSENT FOR PUBLICATION

All authors concur with the submission and publication of this research article.

ADDITIONAL INFORMATION

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Correspondence and requests for materials should be addressed to Zhigang Zhao or Zhiqiang Liu.

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