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ORIGINAL ARTICLE

Gene expression profile analysis of U251 glioma cells with shRNA-mediated SOX9 knockdown

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Summary

Purpose: *In glioma, the sex-determining region Y-box 9 gene (SOX9) is overexpressed and its downregulation leads to inhibition of cell proliferation, invasion and increased cell apoptosis. To further evaluate the molecular and signal pathways associated with the function of SOX9 and SOX9 target genes, a global gene expression profile of the established SOX9-knockdown U251 cells was investigated.*

Methods: *The molecular function and biological pathways of differentially expressed genes (DEGs) were identified by gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. The interactome networks of DEGs were constructed using the STRING online tool. The genes were further validated by RT-qPCR.*

Results: *GO analysis revealed that a set of 194 DEGs was shared in both the SOX9 KD-1 and SOX9 KD-2 U251 cells. GO analysis and KEGG pathway analysis showed that the DEGs were associated with biological processes involving cellular responses to hypoxia, osteoblast differentiation and angiogenesis, and special biological pathways, such as a TGF-beta signaling pathway and a HIF-1 signaling pathway. In addition, computational network of novel identified potential target genes linked to SOX9, including TGFB2, VEGFA, EGLN3 (PHD3), CA9 and HIF-1a. All of these genes were downregulated in the SOX9 knockdown U251 cells.*

Conclusions: *SOX9 may be a key regulator impacting the glioma cellular processes by influencing the cellular response to hypoxia and HIF-1 signaling pathway. TGFB2, VEGFA, EGLN3 (PHD3), CA9, and HIF-1a may be the target genes of SOX9.*

Key words: *gene expression profile, glioma, hypoxia signaling pathway, SOX9*

Introduction

The sex-determining region Y-box 9 (SOX9) is a member of the SOX family, belonging to the High Mobility Group (HMG) superfamily [1,2]. SOX9 has been shown to play central roles in the regulation of normal embryogenesis, the development of male sex gonad or respiratory epithelium, neural crest's development and differentiation and chondrogenesis [3-7]. Numerous studies have demonstrated that SOX9 is also involved in carcinogenesis in a variety of human cancers, including breast cancer [8], chondrosarcoma [9], prostate cancer [10], gastric carcinoma [11], colorectal cancer [12], and urothe-

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lial carcinoma [13]. In summary, SOX9 functions as an oncogene, enhancing cancer cell growth, angiogenesis and invasion.

Glioma is a refractory and recrudescent cancer, with a high mortality rate and very poor 5-year survival. Researchers have been actively exploring effective therapeutic targets and biomarkers for glioma treatment. SOX9, as an oncogene, is involved in a variety of tumor-associated molecular pathways, and recently it has become a new target for the treatment of glioma. The function and molecular mechanisms of SOX9 in glioma were explored in several studies. Wang et al. [14] found that the mRNA expression level of SOX9 in glioma tissues was significantly higher than that in corresponding non-neoplastic brain tissues. Moreover, SOX9 mRNA expression level increased significantly in glioma tissues with advanced WHO grade. Cao et al. [15] examined the functional roles of SOX9 in glioma using gene set enrichment analysis (GSEA), and showed that SOX9 expression was commonly upregulated in glioma tissues, and patients with high SOX9 levels had shorter survival. Furthermore, SOX9 downregulation decreased cyclin D1, CDK4 expression and Rb phosphorylation, which correlated with reduced cell population in the S phase and suppressed tumor growth. Liu et al. [16] reported that SOX9 stimulated metastasis through activating Wnt/b-catenin signaling. A study by Swartling at al. [17] reported that SOX9 expression was reduced when *Prkg2* gene was consistently transfected. When the glioma cells treated with cGMP analog, GKII was activated, SOX9 was phosphorylated, SOX9 protein expression was suppressed and Akt phosphorylation and G1 arrest were inhibited. In addition, downregulation of SOX9 expression by siRNA was shown to reduce glioma cell proliferation. However, the precise functions and molecular mechanisms of SOX9 in gliomas remain to be elucidated.

In order to further investigate the molecular and signal pathways associated with the function of SOX9 in glioma, we analyzed the global gene expression profiles in U251 glioma cells and the corresponding U251 cells in which SOX9 was downregulated by lentivirus-mediated short hairpin RNA (shRNA).

Methods

Samples

Human glioma cell line U251 was purchased from the China Science Academy, Shanghai, China, and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco®; Invitrogen) at 37 °C, in 5% CO_2 humidified atmosphere. SOX9 knock-down U251 cells were generated by transfecting U251 cells with lentivirus-mediated SOX9-shRNA1 and shRNA2. Control cells (NC) were generated by transfecting U251 cells with NC-shRNA. The SOX9 knockdown (KD) U251 cells (U251 KD-1, U251 KD-2) and control U251 cells (U251 NC) were confirmed with identification and screening by qRT-PCR.

Gene chip and expression profiling

The assessment of RNA integrity is a critical first step in obtaining meaningful gene expression data. Total RNA was isolated from U251 KD-1, U251 KD-2 and U251 NC cells and purified using QIAGEN RNeasy® Mini Kit. Total RNAs of each condition were quantified by the NanoDrop ND-2000 (Thermo Scientific, Waltham, MA,USA) and the RNAs integrity was assessed using Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Based on the manufacturer's standard protocols, the samples were labeled, washed and microarray hybridization was conducted. In brief, total RNAs were transcribed to double strand cDNAs and then synthesized cRNAs. Next, the 2nd cycle cDNAs were synthesized from cRNAs. Following fragmentation and biotin labeling, the 2nd cycle cDNAs were hybridized onto the microarray. After washing and staining, the arrays were scanned by the Affymetrix Scanner 3000 (Affymetrix, Santa Clara, CA, USA). The total RNA was sent for global gene expression profile analysis using an Affymetrix HTA 2.0 gene chip (Shanghai Biotech Co., Ltd., Shanghai, China). Affymetrix Gene Chip Command Console (version 4.0, Affymetrix) software was used to extract the raw data. Next, Expression Console (version1.4.1,Affymetrix) software offered SST-RMA normalization for both the gene and exon levels analysis. The standardized gene level data contained standardized signal value and detailed annotation information. Genespring software (version 13.1; Agilent Technologies) was utilized to finish the basic analysis.

Bioinformatic analysis of ChIP data

DEGs were identified through fold change of the expression. Changes in gene expression with a 2-fold change were considered differentially regulated by SOX9. The screening of DEGs compared the SOX9 knockdown U251 cells (SOX9 KD-1 and SOX9 KD-2) with the U251 NC cells. Functional classification, biochemistry pathway and biological annotation were used to clarify the biological process of SOX9 regulated genes. GO analysis and KEGG analysis were applied to determine the roles of these differentially expressed mRNAs displayed in these GO terms or pathways. GO were analyzed and visualized using http://geneontology.org and visualization tool Amigo2 (http://amigo. geneontology.org/visualize?mode= client_ amigo).

Network analysis

The set of 200 genes most upregulated (n=100) or downregulated (n=100) in the SOX9 KD-1 and SOX9 KD-2 U251 cells was uploaded into STRING (http://string-db.org/) and the networks were evaluated. The minimum required interaction score was set to high confidence

(0.700) and the active interaction sources used were text-mining, experiments, databases, neighbourhood, gene fusion, co-occurrence and co-expression. Figures of the networks, and quantitative network characteristics and gene ontologies were downloaded.

RNA isolation, reverse transcription and real-time PCR

Total RNA was isolated using Mini BEST^{T} Universal RNA Extraction Kit (Takara), and cDNA was synthesized with a Prime Script™ RT reagent kit (Takara). DEGs were selected and tested by quantitative real-time (qRT-PCR). The primer sequences for the DEGs used in this study are listed in Table 1. GAPDH was used as a reference gene. The qRT-PCR was performed using SYBR® Primix Ex TaqTM (Takara). PCR amplification was performed in a total volume of 20 μL including 1 μL of cDNA, 0.5 mol/L of each primer, and 10 μL of SYBR Green Real-time PCR Master Mix. qRT-PCR was conducted on Applied Biosystems StepOne Fluorescent Quantitative PCR (Applied Biosystems Inc., ABI, Foster City, CA, USA).

Statistics

Statistical analyses were performed using commercially available software (SPSS version 16, IBM Corp.). The independent samples t-test analysis was used to determine the relative mRNA expression level of the validated DEGs. Differences were considered statistically significant at $p<0.05$ and $p<0.01$. Graphs were performed by Prism GraphPad Software (Version 5.0C, San Diego, CA), and their values were presented as mean±SD.

Results

Global gene expression profile in SOX9 knockdown U251 cells

Global gene expression was analyzed by comparing the transcriptome profiles of the SOX9 KD-1 and SOX9 KD-2 U251 cells with U251 NC cells. The selection of DGEs was performed through a simple fold change cutoff (reference parameter values were ≥2.0 fold changes in our experiments). Comparing with U251 NC cells, there were 5596 DEGs in U251 KD-1 cells. Among these, 2870 DEGs were upregulated, and 2726 DEGs were downregulated. Besides, there were 442 DEGs between SOX9 KD-2 U251 cells and control U251 cells, and 221 DEGs were upregulated and downregulated, respectively. A set of 194 DEGs was shared both in SOX9 KD-1 and SOX9 KD-2 U251 cells, shown by Venn picture (Figure 1).

Table 1. Primer sequence for real-time polymerase chain reaction

Gene symbol	*Forward(5'-3')*	*Reverse(5'-3')*	*Size(bp)*
TGFB2	CTTTGGATGCGGCCTATTGC	TCCAGCACAGAAGTTGGCAT	137
VEGFA	CCAGCAGAAAGAGGAAAGAGGTAG	CCCCAAAAGCAGGTCACTCAC	133
PHD3	GGCTGGGCAAATACTACGTCAA	CCTGTTCCATTTCCCGGATAG	70
HIF-1a	GATAGCAAGACTTTCCTCAGTCG	TGGCTCATATCCCATCAATTC	90
CA9	TTTGCCAGAGTTGACGAGGC	GCTCATAGGCACTGTTTTCTTCC	97
SOX9	AGGTGCTCAAAGGCTACGACT	AGATGTGCGTCTGCTCCGTG	360
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	226

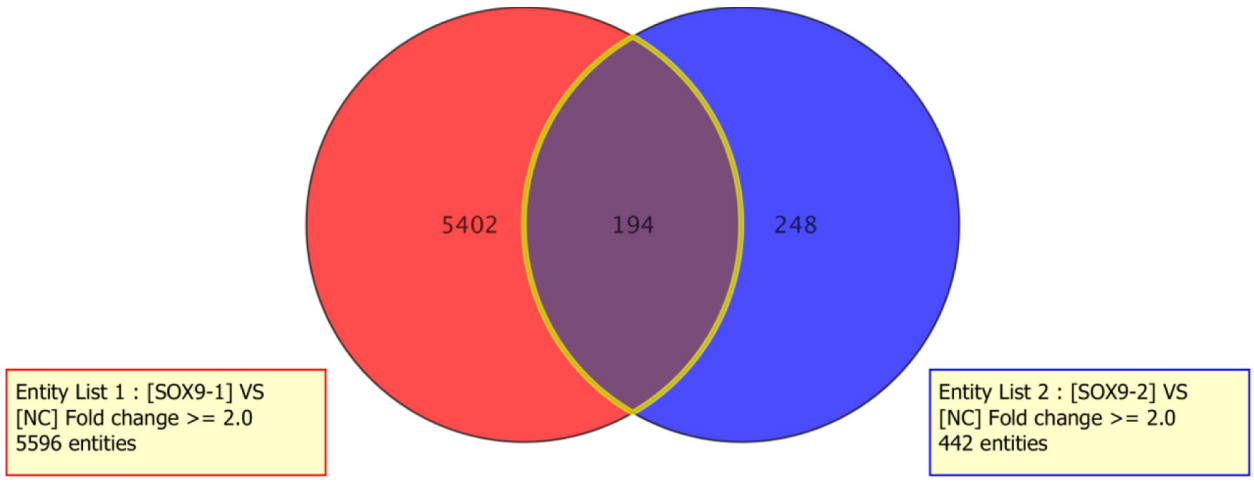

Figure 1. Venn diagram of screening differential expressed genes (DEGs) analysis. The total DEGs of SOX9 KD-1 U251 cells were 5596, which are shown in the red circle. The total DEGs of SOX9 KD-2 U251 cells were 442, which are shown in the blue circle. The common DEGs were 194

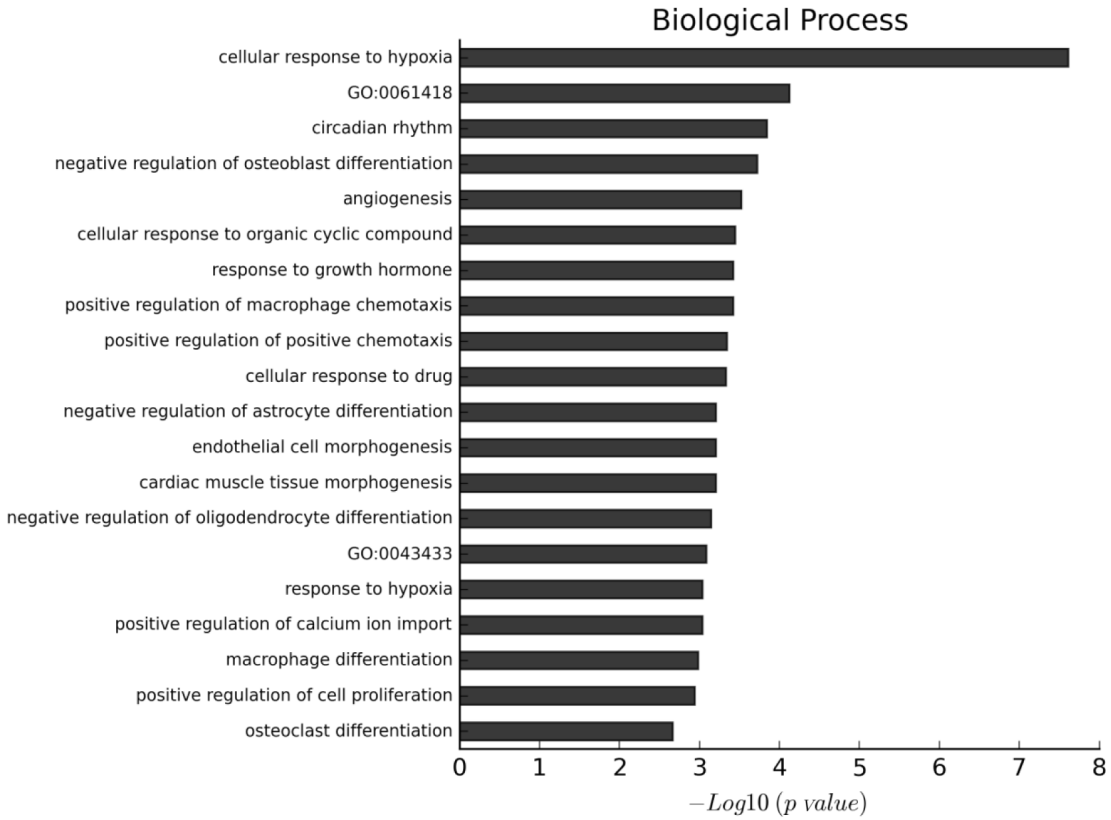

Figure 2. Gene ontology (GO) enrichment analysis for differentially regulated genes. GO analysis of genes according to biological process. The top 20 GO terms of DEGs between SOX9 downregulated U251 cells and NC U251 cells are shown in this histogram.

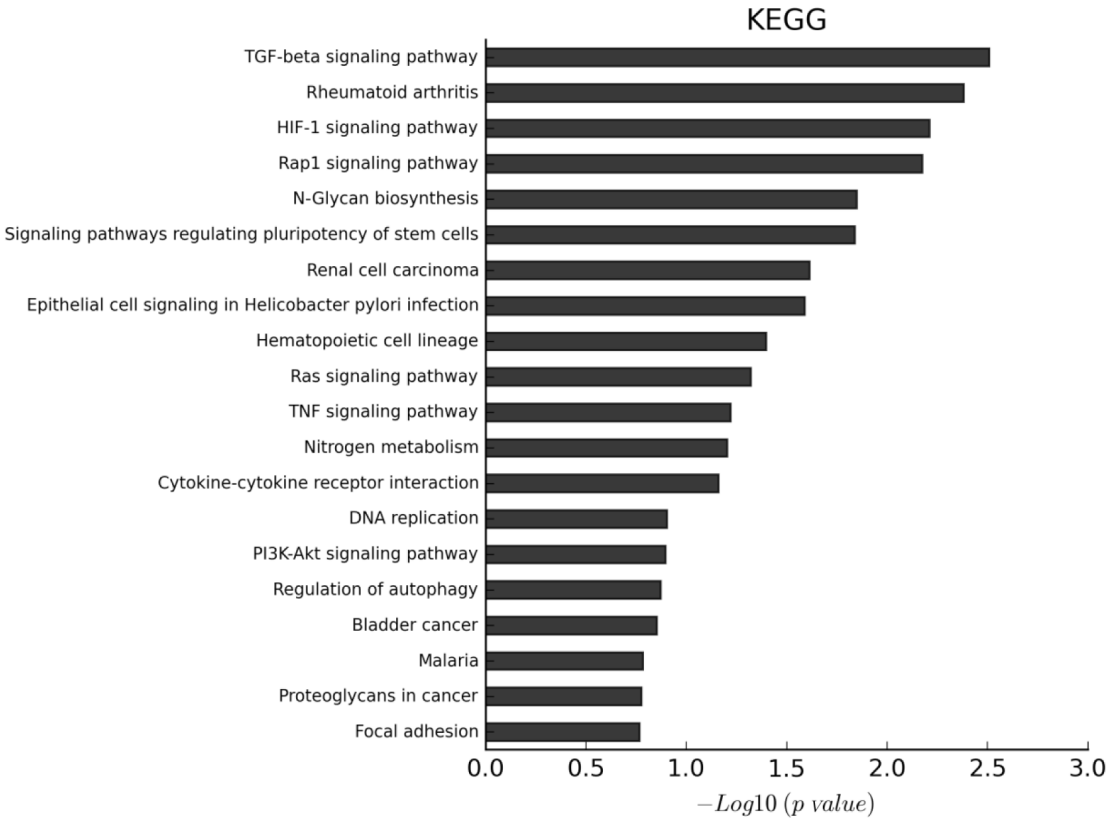

Figure 3. KEGG pathway enrichment analysis for differentially expressed genes (DEGs). The top 20 pathways of DEGs between SOX9 downregulated U251 cells and NC U251 cells are shown in the histogram.

Bioinformatic analysis of SOX knockdown U251 cells

In order to investigate the molecular function and biological pathways of the DEGs, SOX9-targeted genes were mapped onto GO analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

We analyzed the enrichment of DEGs (Table 2). The result showed that the most significant functional group of biological process was cellular response to hypoxia (Figure 2). A ≥2.0-fold change and a p value <0.05 were considered significant. The top 10 enriched GO terms of DEGs were selected and are shown in Table 2. The main enriched GO terms of DEGs were associated with biological processes involving cellular response to hypoxia, osteoblast differentiation, and angiogenesis. The most related genes were CA9, EGLN3 and VEGFA.

We next performed KEGG pathway analysis of DEGs and the top 10 enriched pathways are shown in Table 3. Significantly enriched KEGG terms of DEGs were observed (Figure 3), and the DEGs were more relevant to the TGF-beta signaling pathway and HIF-1 signaling pathway.

The interactome networks of DEGs of the SOX9 knockdown U251 cells

In order to better understand the interaction of DEGs and to discover further potential therapeu-

Table 2. Detailed information of biological process

Term ID	*Term description*	*List Hit*	*List Total*	*Gene symbols*	*p value*	*FDR_ bh*
GO:0071456	cellular response to hypoxia	7	44	NDRG1;CA9;EGLN1;STC1; PLAU;VEGFA;ANKRD1	2.44E-08	1.11E-05
GO:0061418	regulation of transcription from RNA polymerase II promoter in response to hypoxia	3	44	CA9;EGLN1;VEGFA	7.53E-05	0.017159
GO:0007623	circadian rhythm	4	44	ID4;ID3;ID1;NTRK3	0.000143	0.021285
GO:0045668	negative regulation of osteoblast differentiation	3	44	ID3;ID1;IGFBP5	0.000188	0.021285
GO:0001525	angiogenesis	5	44	TGFBI;PLAU;VEGFA;ID1;CCL2	0.000297	0.021285
GO:0071407	cellular response to organic cyclic compound	3	44	IGFBP5;CCL2;ANKRD1	0.000356	0.021285
GO:0060416	response to growth hormone	2	44	TRIM16;IGFBP5	0.000376	0.021285
GO:0010759	positive regulation of macrophage chemotaxis	2	44	CSF1;CCL2	0.000376	0.021285
GO:0050927	positive regulation of positive chemotaxis	2	44	VEGFA;NTRK3	0.00045	0.021285
GO:0035690	cellular response to drug	3	44	TFRC;CCL2;ANKRD1	0.000466 778	0.021285 701

Table 3. Detailed information of KEGG pathway analysis

Term ID	*Term description*	*List Hit*	*List Total*	*Gene symbols*	*p value*	*FDR_ bh*
path:hsa04350	TGF-beta signaling pathway	3	25	ID4;ID1;ID3	0.003096	0.096885
path:hsa05323	Rheumatoid arthritis	3	25	VEGFA;CSF1;CCL2	0.004143	0.096885
path:hsa04066	HIF-1 signaling pathway	3	25	EGLN1;TFRC;VEGFA	0.006158	0.096885
path:hsa04015	Rap1 signaling pathway	4	25	PDGFD;VEGFA;ID1;CSF1	0.006682	0.096885
path:hsa00510	N-Glycan biosynthesis	2	25	ALG5;GANAB	0.014132	0.140074
path:hsa04550	Signaling pathways regulating pluripotency of stem cells	3	25	ID4;ID1;ID3	0.01449	0.140074
path:hsa05211	Renal cell carcinoma	2	25	EGLN1;VEGFA	0.024331	0.186199
path:hsa05120	Epithelial cell signaling in Helicobacter pylori infection	2	25	PTPRZ1;GIT1	0.025683	0.186199
path:hsa04640	Hematopoietic cell lineage	2	25	TFRC;CSF1	0.039962	0.257531
path:hsa04014	Ras signaling pathway	3	25	PDGFD;VEGFA;CSF1	0.047748	0.276937

tic targets, we constructed interactome networks of DEGs using the STRING online tool. From our DEGs database we extracted the 50 most-highly expressed genes and the 50 most-highly inhibited genes when gene expression from the SOX9 down-regulation condition was compared to the normal control condition. The resulting networks are illustrated in Figure 4. In networks, TGFB2, VEGFA, EGLN3, CA9 were strongly associated with SOX9. This network included genes already known to be of importance in glioma biology, e.g. TGFB2, VEGFA. The combined network also indicated novel genes that ought to be explored as future potential targets for the treatment of glioblastoma multiforme.

Table 4. Detailed chip information of genes related to hypoxia and associated with SOX9 in interactome network

Probe Set ID	*Fold change*	*Regulation*	*Gene symbol*	*Gene description*	*p value*
TC01001805.hg.1	-2.4366624	down	TGFB2	transforming growth factor, beta 2	0.004
TC14001022.hg.1	-5.3006454	down	EGLN3(PHD3)	egl nine homolog 3 (C. elegans)	0.084
TC06000608.hg.1	-2.1782002	down	VEGFA	vascular endothelial growth factor A	0.032
TC17000819.hg.1	-8.301469	down	SOX9	SRY (sex determining region Y)-box 9	0.002
TC09000191.hg.1	-190.23251	down	CA9	carbonic anhydrase IX	0.002

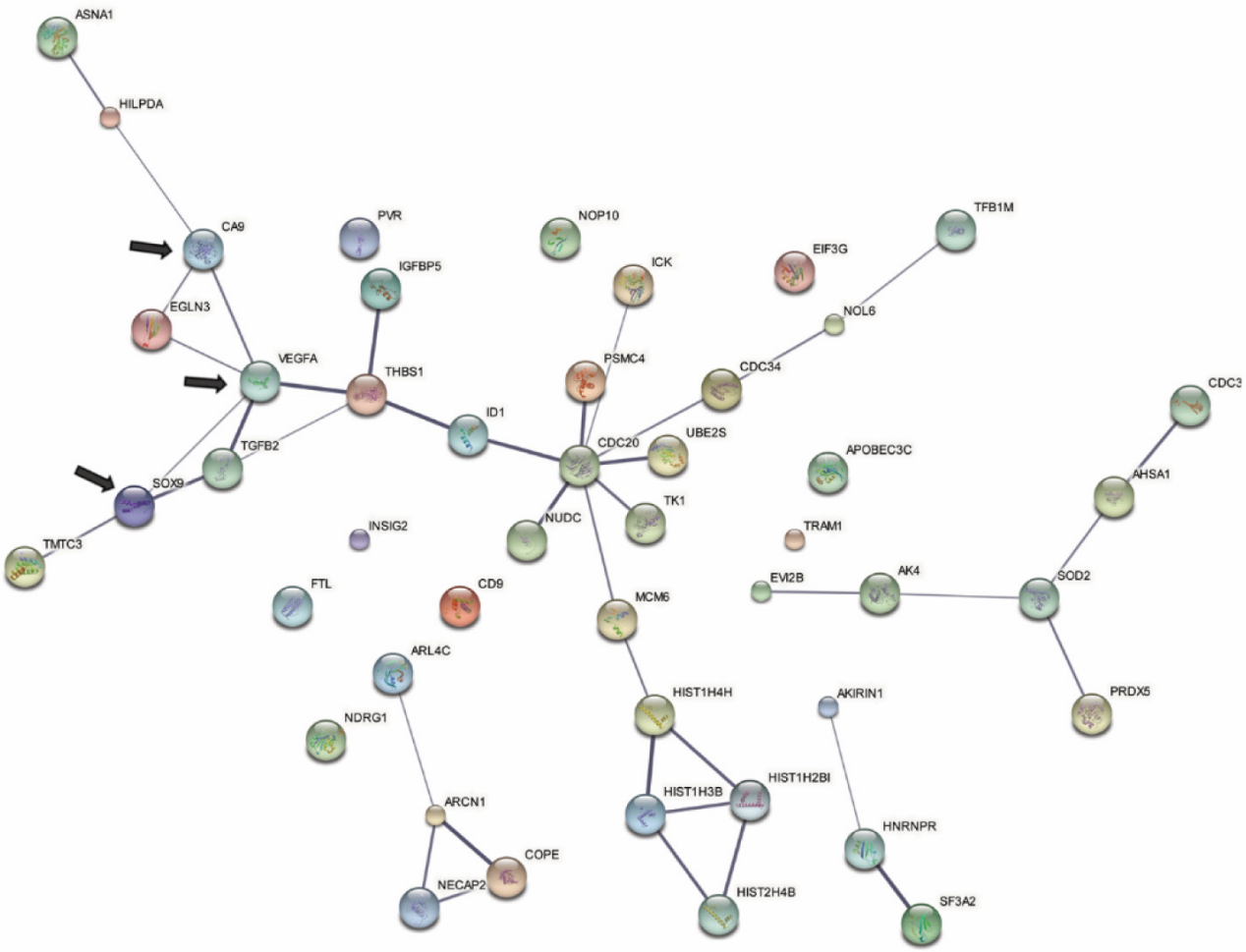

Figure 4. The interactome network of the common differentially expressed genes (DEGs). Network nodes represent protein; edges represent protein-protein associations. Note that SOX9 was very strongly associated with VEGFA and TGFB2, and further related with CA9 and EGLN3 (also known as PHD3).

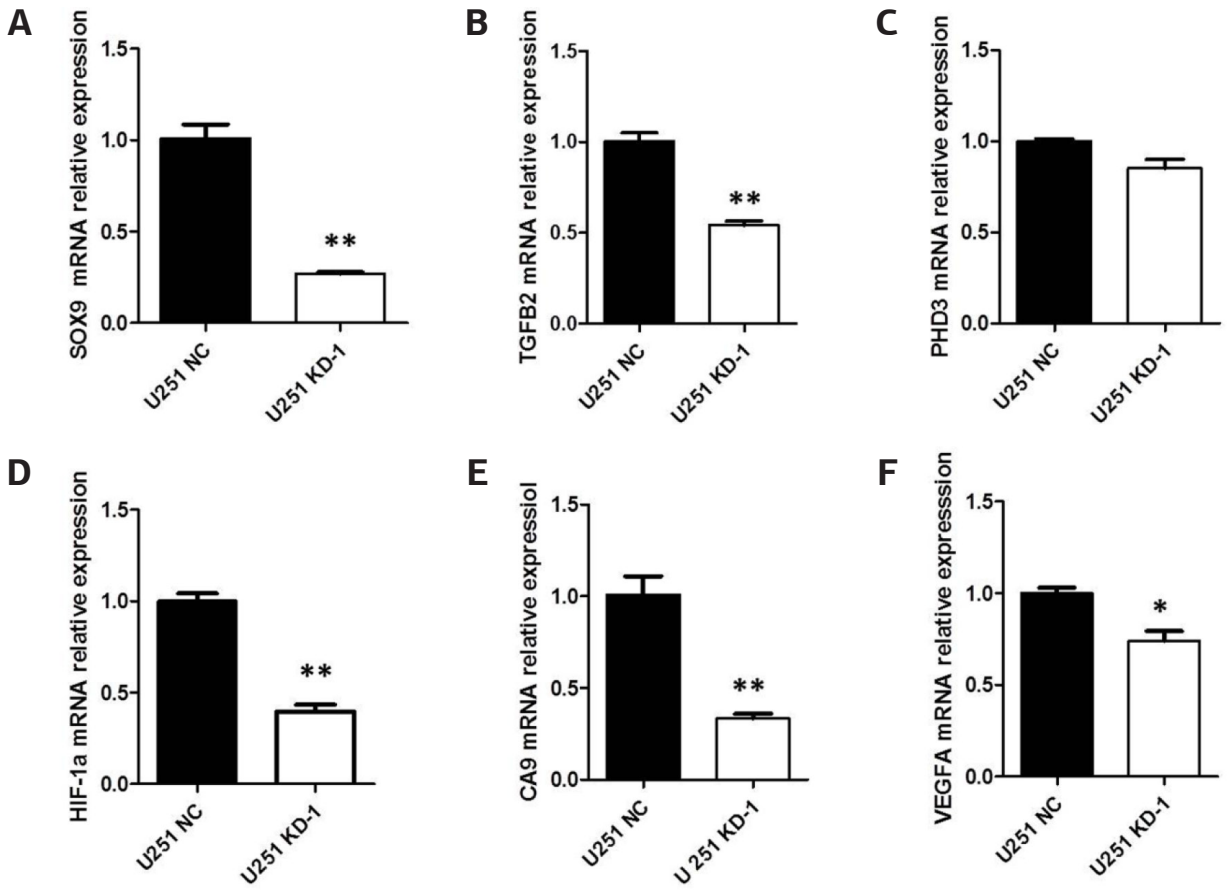

Figure 5. Validation of genes' expression level in the SOX9 knockdown U251 cells and U251 control cells by qRT-PCR. **(A)** shows that SOX9 was significantly knocked down in U251 KD-1. The mRNA expression level of TGFB2 **(B)**, PHD33 **(C)**, HIF-1a **(D)**, CA 9 **(E)** and VEGFA **(F)** were upregulated in SOX9 knockdown U251 cells. *p<0.05, **p<0.01.

Very little information is currently known about the activities of these gene products in glioma biology. The genes shown in Table 4 correspond to the networks in Figure 4. The detail GO illustrate the close correlation of the function of the genes involved with SOX9 in glioma, all of which were enriched in the SOX9 knockdown U251 cell lines.

Expression of TGFB2, EGLN3, VEGFA, CA9 and HIF-1a in SOX9 knockdown U251 cells

After obtaining interesting data from the gene expression profile analysis, we selected and examined the expression levels of TGFB2, EGLN3 (PHD3), VEGFA, CA9 and HIF-1a. The expression of the genes was calculated according to the relative qRT-PCR formula $2^{-\Delta\Delta Ct}$ • 100%. The fold change ratios were determined to be between the SOX9 knockdown U251 cells and control U251 cells. The qRT-PCR expression pattern of selected DEGs listed in Table 4 were in agreement with the chip analysis. The expression level of the TGFB2, EGLN3 (PHD3), VEGFA, CA9 and HIF-1a were downregulated in the SOX9 downregulation U251 cells, compared with the control U251 cells. All these genes, except EGLN3 (PHD3; p>0.05), were significantly downregulated in the SOX9 downregulated U251 cells (p<0.05, Figure 5).

Discussion

Glioblastoma represents the most frequent and most malignant brain tumor [18]. Despite great efforts to improve treatment, the median overall survival rate of patients treated with standard therapies (radical resection, postoperative radiotherapy and sequential temozolomide chemotherapy) is still below 15 months [19]. The development of malignant cells in a tumor generally needs to sustain proliferative signaling, enable replicative immortality, restrain cell death, induce angiogenesis, and promote invasion and metastasis [20]. Molecules and signal pathways involved into those processes may be targets to inhibit and cure malignant tumors.

SOX9 is involved in the regulation of a variety of tumor-related molecular pathways, including EGFR [13], Notch [21], WNT [22], Sonic hedgehog [23], hypoxia [24] and a variety of downstream mo-

lecular pathways, including AKT [25], WNT [26] and BMI1 [27]. However, to date, only a few genes have been shown to be SOX9 targets, while little is known about SOX9-independent regulators. Current studies have shown that the occurrence of glioma is not the result of a single signal pathway change, but a complex tumor-related molecular network regulation. In order to search for these important molecules, we used gene chip and bioinformatics analysis to study the gene expression profiles of glioma cell line U251 after knockdown of SOX9 by DEGs screening, GO analysis, KEGG pathway analysis and protein interaction analysis.

We performed Transcriptome Array for three samples (U251 KD-1, U251 KD-2 and U251 NC cells). The efficiency of SOX9 knockdown in U251 KD-1 cells was higher than U251 KD-2 cells compared with the baseline SOX9 expression level of U251 NC cells (data not shown). Thus, much more DEGs were selected from the U251 KD-1 cells. The common DEGs set shrunk the scope of the study, making the next step of analysis more accurate. GO provides core biological knowledge for modern biologists, either computationally or experimentally based. In the chip data analysis, the researchers can find out which genes belong to a common GO term, and test whether the results are statistically significant by statistical methods. Thus, it can be concluded that DEGs mainly belong to specific biological functions. In our research, the main enriched GO terms of DEGs were associated with biological processes involving cellular response to hypoxia, osteoblast differentiation and angiogenesis. In addition, the most related genes included CA9, EGLN3 and VEGFA. Based on the selected DEGs, the hypergeometric distributions of these DEGs were calculated. The KEGG pathway analysis returned a p value for the pathway of each differential gene. A small p value indicated that the differential gene was present in the pathway enrichment. Pathway analysis of the results suggests that the role of the pathogenesis of the differential gene pathways can be found in the enrichment differential gene pathway entries. Different samples of different genes may be associated with changes in cellular pathways. The results showed that the DEGs were more relevant to the TGF-beta and HIF-1 signaling pathway. To study the interaction of SOX9 and other proteins, protein network analysis was performed. Researchers can gain more in-depth understanding of protein function and a clearer understanding of its regulatory mechanism. The results showed that TGFB2, VEGFA, EGLN3, CA9 were strongly associated with SOX9 and they may be the key regulators of SOX9 regulated signaling pathways.

Hypoxia is known to represent a crucial step in the development of aggressive malignant features of many human cancers. Hypoxia-induced activation of the HIF-1 pathway is an essential characteristic of malignant gliomas [28]. Hypoxia has been linked to tumor progression, therapy resistance and poor prognosis [29]. The transcription factor hypoxia inducible factor-1 (HIF-1), a dimer of HIF-1a and HIF-1b, is a critical mediator of response to hypoxia [30,31]. HIF-1α is a unique oxygen-regulated component, which determines HIF-1 activity, such as induction of the expression of genes related to tumor angiogenesis, cell proliferation and metabolism [32,33]. Upregulation of HIF-1a expression level can promote tumor growth, and inhibition of HIF-1a activity can dramatically decrease tumor growth, vascularization and energy metabolism [34]. SOX9 also plays an important role in the oxygen-independent regulation of hypoxia-associated gene expression responsible for tumor progression and metastases. Zhang et al. [35] reported that HIF-1α activates SOX9 expression and enhances SOX9-mediated transcriptional activity and that HIF-1α physically interacts with SOX9. Similarly, Amarilio et al. [36] demonstrated that HIF-1a can bind directly to the SOX9 promoter, thus supporting direct regulation of HIF-1a on SOX9 expression. However, a study of Camaj et al. [37] revealed that SOX9 expression does not seem to interfere with HIF-1α gene regulating machinery. SOX9 activation seems to have a stronger effect on the transcriptome and therefore can overcome hypoxic HIF-1α–based hypoxic regulation. The interaction between HIF-1a and SOX9 remains controversial. HIF-1a expression level was downregulated in SOX9 knockdown U251 cells in this study (Figure 5) and the mechanism needs to be further searched.

Vascular endothelial growth factor A (VEGFA) was a pivotal regulator of angiogenesis and played an important role in tumor proliferation, angiogenesis and metastasis [38]. VEGFA was specifically bound to VEGFR-1 and VEGFR-2 [39]. VEGFA and its receptor have been the research focus on antiangiogenic therapy [40]. Hattori et al. [41] showed that SOX9 has the capacity to downregulate VEGFA gene transcription by direct interaction with regulatory SRY elements in the VEGFA gene. SOX9 is a direct suppressor of VEGFA expression, ectopically upregulated at both the mRNA and protein levels [42]. In addition, research has shown that VEGF is a direct target of HIF-1α [43] and the stabilization of HIF1-α can increase VEGFA expression [44]. Knockdown of SOX9 can lead to restoration of HIF-1α–driven hypoxic inducibility of the downstream target genes, suggesting that an overlap of activity between SOX9 and VEGF exists.

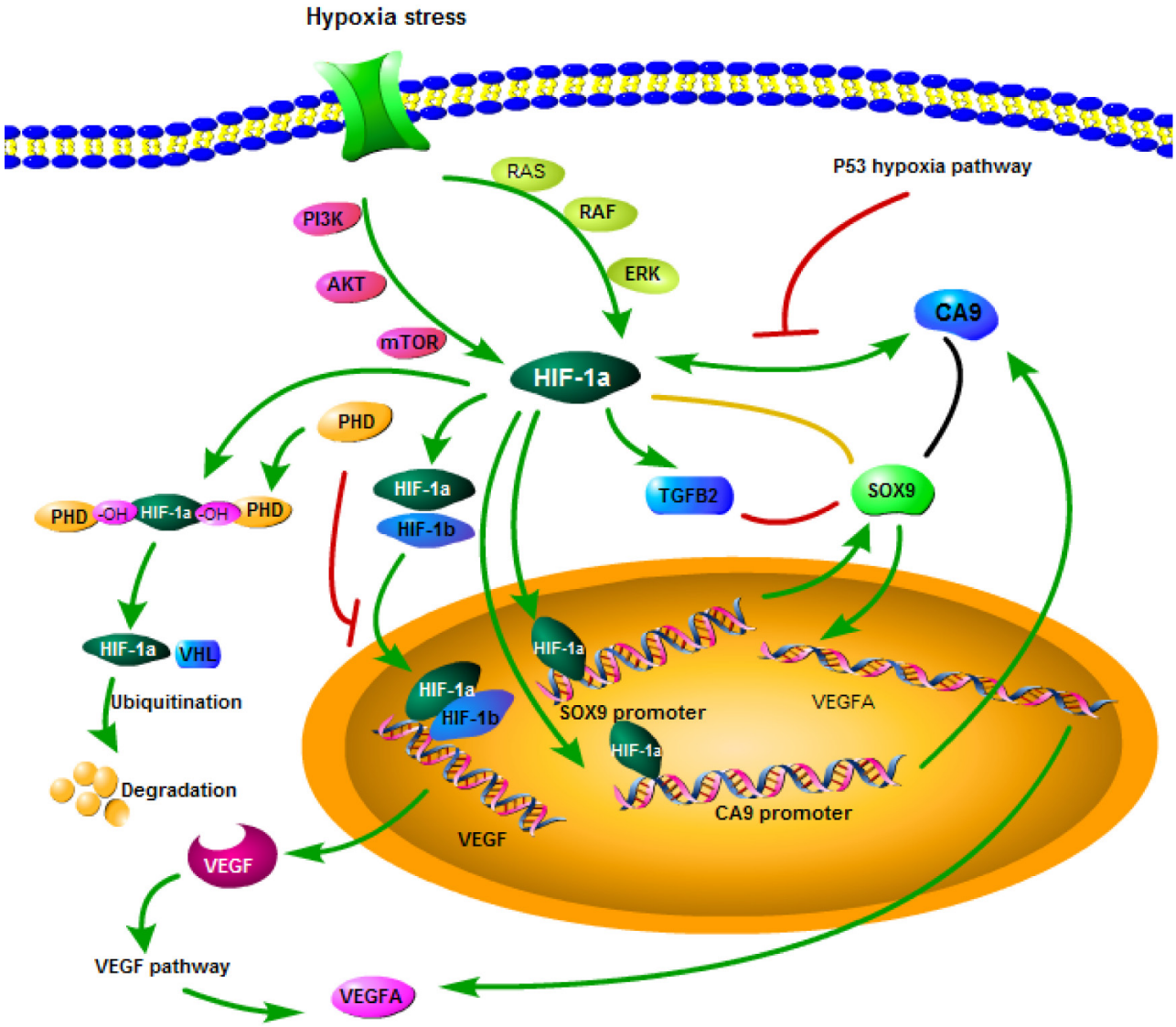

Figure 6. Schematic model of the molecular mechanism of SOX9 regulated genes. The schematic figure was made in Portable Pathway Builder tool 2.0. The Figure shows that hypoxia and HIF-1a, VEGFA, TGFB2, CA9 and PHD interacted directly or indirectly with SOX9 in mRNA and protein level.

TGF-β is the prototypic member of the TGF-β superfamily of growth factors [45]. Three mammalian isoforms of TGF-β (1–3) exist and while their active forms mostly have similar actions on cells *in vitro*, they also have non-redundant functions *in vivo* [46,47]. This highly pleiotropic growth factor plays a key role in various cellular processes, including inhibition of cell growth, induction of apoptosis and production of extracellular matrix proteins directly or indirectly by modulating TGF-β signaling. TGF-β2 is unique among the three isoforms in that it lacks a RGD integrin-binding sequence in its precursor and is the only isoform with multiple CRE elements immediately upstream of its P1 promoter. Of note, the CREB family of transcription factors has recently been shown to be activated by hypoxia [48]. Hypoxic markers (PHD3, HAS2, VEGF, COX2) as well as SOX9 were upregulated in hypoxic culture, while simultaneously TGFB2 was upregulated under hypoxia on both the gene and protein level [49]. Gris et al. [50] reported that TGFB2, in combination with SOX9, can differentially regulate the expression of XT-I, XT-II and C4ST in primary astrocytes. In our study, both chip data and qRT-PCR showed that TGFB2 was decreased in SOX9-downregulated U251 cells, and that there was no direct evidence to identify the relationship about TGFB2 and SOX9 in previous studies.

The prolyl hydroxylase domain protein 3 (PHD3) was also known as EGLN3, HIF-P4H3, or HPH1 [51]. PHD3 upregulation has also been linked to apoptosis in carcinoma cells, where apoptosis correlated with aggregate formation [52]. Decreased PHD3 mRNA levels and a lack of hypoxic

upregulation have been reported in human prostate and breast carcinoma cell lines, which have mechanically been linked to aberrant PHD3 promoter methylation [53,54]. Similarly, to normal neuronal cells, in the development of neuroblastomas, PHD3 has been proposed to eliminate excess neurons by regulating KIF1B, and the loss-of-function mutations of KIF1B have been linked to the formation of tumors [55]. Furthermore, PHD3 overexpression is found in pancreatobiliary and pancreatic cancers, where PHD3 may be upregulated more than 10-fold, most notably in well-differentiated tumors, although metastatic lesions were reported to exhibit lower PHD3 [56]. No direct interaction between PHD3 and SOX9 has been reported.

CA9 is a transmembrane zinc metalloenzyme functioning as the catalyst of reversible hydration of carbon dioxide to bicarbonate ions and protons [57]. The protein belongs to a class of the family of carbonic anhydrases (CAs) expressed in higher vertebrates [58]. Accumulating experimental evidence supports the direct participation of CA9 in many hypoxia- and acidosis-induced features of tumor phenotype including increased adaptation of tumor cells to microenvironmental stresses, resistance to therapy, increased tumor cell migration and invasiveness, increased focal adhesion during cell spreading, destabilization of intercellular contacts, maintenance of stem cell phenotype, tumor-stroma crosstalk, signal transduction and possibly other cancer-related phenomena [59]. CA9 was shown to be involved in this phenomenon in breast and esophageal carcinoma models [60,61]. Expression of CA9 correlates with the expression of a stem cell marker CD44 [62,63] and CA9 targeting by inhibition of its catalytic activity results in inhibition of breast cancer stem cell expansion in hypoxia, indicating that pH-regulating function of CA9 is required for the maintenance of stemness [64].

Both CA9 and PHD3 were decreased in SOX9-shRNA treated U251 cell in our research, while no interaction between these two proteins and SOX9 had be seen in PubMed. Whether there exists an indirect relationship needs to be further investigated, and HIF-1a may be a core protein. Kessler et al. [29] showed that U251MG and U343MG exhibited a weak HIF-1a protein expression under normoxia with an increase in HIF-1a protein, CA9 mRNA and CAIX protein levels under hypoxia. Monitoring CA9 mRNA and CAIX protein expression is a mean to verify the inhibition of HIF-1 activity. HIF-1a was identified as an endogenous marker of tumor hypoxia [65,66]. Furthermore, CAIX was found to be a suitable marker of current or previous chronic hypoxia [67,68]. A study by Dai et al. [28] found that the presence of wild type (wt) p53 under hypoxic conditions has an insignificant effect on the stabilization of HIF-1a protein and HIF-1-dependent expression of CA9. However, upon activation by DNA damage, wtp53 mediates an accelerated degradation of HIF-1a protein, resulting in reduced activation of CA9 transcription and, correspondingly, decreased the levels of CA9 protein. PHD3 has been shown to catalyze the proteolytic degradation of HIF-1a and inhibition of VEGF gene expression [69]. HIF expression and transcriptional activity are regulated by the oxygen-sensitive prolyl hydroxylases (PHD1–3) [70].

In conclusion, SOX9 may be a key regulator impacting the glioma cellular processes by regulating cellular response to hypoxia and HIF-1 signaling pathway, and TGFB2, VEGFA, EGLN3 (PHD3), CA9, and HIF-1a may be SOX9 target genes. All these genes interact with each other playing an important role in the function of SOX9 (Figure 6), but the meticulous study of mechanism was incomplete and needs further exploration.

Authors' contributions

Yanyang Tu and Liang Wang conceived and designed most experiments. Dongni Ren and Chen Yang developed the methodology. Dongni Ren and Chen Yang performed most experiment. Nan Liu, Zhen Wang, Xin Wang and Mark Johnson did the analysis and interpretation of data. Dongni Ren, Chen Yang, Wenshi Wang, wrote, reviewed, and revised the manuscript. Pengxing Zhang, Hongwei Yang, Hui Liu, and Yingduan Cheng supported study supervision.

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Conflict of interests

The authors declare no conflict of interests.

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