

## Basic Study

Long non-coding RNA *SNHG16* promotes human placenta-derived mesenchymal stem cell proliferation capacity through the PI3K/AKT pathway under hypoxia

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## Abstract

## BACKGROUND

The effect of hypoxia on mesenchymal stem cells (MSCs) is an emerging topic in MSC biology. Although long non-coding RNAs (lncRNAs) and messenger RNAs (mRNAs) are reported to play a critical role in regulating the biological characteristics of MSCs, their specific expression and co-expression profiles in human placenta-derived MSCs (hP-MSCs) under hypoxia and the underlying mechanisms of lncRNAs in hP-MSC biology are unknown.

## AIM

To reveal the specific expression profiles of lncRNAs in hP-MSCs under hypoxia and initially explored the possible mechanism of lncRNAs on hP-MSC biology.

## METHODS

Here, we used a multigas incubator (92.5% N<sub>2</sub>, 5% CO<sub>2</sub>, and 2.5% O<sub>2</sub>) to mimic the

hypoxia condition and observed that hypoxic culture significantly promoted the proliferation potential of hP-MSCs. RNA sequencing technology was applied to identify the exact expression profiles of lncRNAs and mRNAs under hypoxia.

## RESULTS

We identified 289 differentially expressed lncRNAs and 240 differentially expressed mRNAs between the hypoxia and normoxia groups. Among them, the lncRNA *SNHG16* was upregulated under hypoxia, which was also validated by reverse transcription-polymerase chain reaction. *SNHG16* was confirmed to affect hP-MSC proliferation rates using a *SNHG16* knockdown model. *SNHG16* overexpression could significantly enhance the proliferation capacity of hP-MSCs, activate the PI3K/AKT pathway, and upregulate the expression of cell cycle-related proteins.

## CONCLUSION

Our results revealed the specific expression characteristics of lncRNAs and mRNAs in hypoxia-cultured hP-MSCs and that lncRNA *SNHG16* can promote hP-MSC proliferation through the PI3K/AKT pathway.

**Key Words:** Human placenta-derived mesenchymal stem cell; Hypoxia; Long non-coding RNAs; Proliferation; Mesenchymal stem cell

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**Core Tip:** This study revealed the specific expression and co-expressed profiles of long non-coding RNAs and messenger RNAs in human placenta-derived mesenchymal stem cells under hypoxia by RNA sequencing assays. Through the performance of a series of systemic bioinformatic analyses, the hypoxia-responsive long non-coding RNA *SNHG16* that may play a role in proliferation was screened out. Furthermore, through the use of molecular biology experiments, *SNHG16* was found to affect human placenta-derived mesenchymal stem cell proliferation rates and cell cycle progression by activating the PI3K/AKT pathway and upregulating the expression of the key cell cycle regulators.

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## INTRODUCTION

Recently, mesenchymal stem cells (MSCs) have gained much attention due to their therapeutic effects and potential applications in regenerative medicine[1]. MSCs have recently been shown to have therapeutic efficacy in various disease models and clinical diseases such as liver injury, coronavirus disease 2019, and Crohn's disease[2-5]. MSCs have been reported to be present in bone marrow, placenta, umbilical cord, and adipose tissue and can be efficiently isolated[6-8]. However, the application of MSCs is limited due to the difficulty in obtaining the large numbers of MSCs required for clinical treatment ( $3 \times 10^7$  cells *per* infusion)[9]. Tissue-derived primary MSCs occur in small numbers and require *in vitro* expansion before transplantation. Human placenta-derived MSCs (hP-MSCs) are a ubiquitous type of MSCs with lower immunogenicity and higher proliferative potential compared to bone marrow-derived MSCs, but these advantages may be compromised by inappropriate culture or changes in the *in vitro* environment.

To improve the proliferation potential of hP-MSCs *in vitro*, most researchers use different methods to stimulate the microenvironment of MSCs *in vivo*[10]. Among the proposed approaches to mimic the natural cellular microenvironment, hypoxia has garnered enormous interest. Hypoxia has been observed in different tissue niches, including the placenta (1%-5% O<sub>2</sub>) where hP-MSCs reside[11]. Since the oxygen concentration (almost 21%) in the *ex vivo* culture system is much higher than the physiological oxygen concentration in the body, hypoxia could act as a physiological stimulus with a significant influence on cell fate. Numerous studies have reported that hypoxia can affect various biological properties of MSCs, such as proliferation capacity, multidirectional differentiation potential, migration, and apoptosis[12-14]. However, the underlying molecular mechanisms by which hypoxia regulates MSC biology remain unclear.

Long non-coding RNAs (lncRNAs) are RNAs longer than 200 nt with no protein-coding potential [15]. lncRNAs are the coordinators of the cellular biological regulatory network, participating in a variety of biological and pathological cellular processes such as cellular survival, proliferation, or migration through regulation of gene expression at transcriptional, post-transcriptional, or translational levels [16]. With advancements in gene sequencing technology, more and more lncRNAs related to cellular functions have been identified. However, the impact of hypoxia on the lncRNA expression profile of MSCs remains unclear. In addition, the roles of hypoxia-responsive lncRNAs remain to be explored. In this study, we investigated the effect of hypoxia on the proliferation potential of hP-MSCs and explored the role of lncRNAs in it.

## MATERIALS AND METHODS

### Cell culture

The protocols for hP-MSC isolation and hypoxic culture were as previously described [7]. All protocols for the processing of human tissues and cells were approved by the Ethics Committee of The First Affiliated Hospital of Zhejiang University (No. 2020-1088).

### Colony-forming unit-fibroblast assay

For the colony-forming unit-fibroblast assay, 1000 hP-MSCs were plated on six-well plates in triplicate and cultured in complete medium for 14 d under normoxic or hypoxic conditions with medium changes every 3 d. Colonies were fixed with paraformaldehyde and then stained with crystal violet for enumeration.

### Cell counting kit-8 assay

The corresponding cells were inoculated into 96-well cell culture plates at a density of 2000 cells *per* well. After 24, 48, 72 or 96-h culture, 10  $\mu$ L of cell counting kit-8 reagent (Dojindo, Kumamoto, Japan) was added into each well to incubate for 2 h. The optical density value at 450 nm was measured using a microplate reader.

### Flow cytometry analysis of cell cycle

The cells were collected with trypsin and fixed with cold 70% ethanol for 2 h. Fixed cells were then treated with propidium iodide staining solution (Beyotime, Nanjing, China). The cells were finally analyzed by flow cytometry. The proportions of cell population in G0/G1, S, and G2/M phases of the cell cycle were fitted and calculated using ModFit software.

### Quantitative real-time polymerase chain reaction

Total RNA of cells was obtained using Trizol reagent (Invitrogen, Carlsbad, CA, United States) following the manufacturer's protocol; the concentration of total RNA was quantified using a NanoDrop-2000 (Thermo Fisher Scientific, Waltham, MA, United States). cDNA was synthesized by reverse transcription reaction using a commercial lncRNA quantitative reverse transcription polymerase chain reaction (PCR) Starter Kit (RiboBio, Guangzhou, China). The final relative expression levels of genes were analyzed through the  $2^{-\Delta\Delta Ct}$  method using *GAPDH* as the internal control. Primers were as follows: *GAPDH*: (forward) 5'-ACAACCTTGGTATCGTGGAAGG-3', (reverse) 5'-GCCATCACGC-CACAGTTTC-3'; *SNHG16*: (forward) 5'-GTTGCCACCCACAACCATT-3', and (reverse) 5'-CGCGAGACACCAGGAGAACT-3'.

### Western blot assay

The cellular protein was harvested using RIPA lysis buffer supplemented with protease and phosphatase inhibitor cocktail (Beyotime). The protein concentrations were detected using a BCA kit (Beyotime). The western blot was conducted as previously described [7]. The primary antibodies were anti- $\beta$ -actin (Abcam, Cambridge, United Kingdom), anti-GAPDH (Abcam), anti-hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) (Cell Signaling Technology, Danvers, MA, United States), anti-c-MYC (Abcam), anti-proliferating cell nuclear antigen (Abcam), anti-CDK2 (Abcam), anti-CDK4 (Abcam), anti-CDK6 (Abcam), anti-CyclinD1 (Abcam), anti-CyclinE1 (Abcam), anti-AKT (Abcam), and anti-phospho-AKT (Abcam).

### RNA sequencing

Whole-transcriptome sequencing was quantitatively analyzed by Oebiotech (Shanghai, China). The libraries [including lncRNA and messenger RNA (mRNA)] were generated using TruSeq Stranded Total RNA with Ribo-Zero Gold (Illumina, San Diego, CA, United States) according to the manual. RNA was then sequenced on a HiSeq 2500 instrument (Illumina). Differential expression analysis of lncRNA and mRNA between the hypoxic and normoxic groups was conducted using the DESeq software package. The differentially expressed genes were identified with the criteria of fold change > 1.5 and  $P < 0.05$ . The

Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed for differentially expressed mRNAs to explore their biological functions. Association analysis between lncRNAs and transcription factors and lncRNA-mRNA co-expression analysis were conducted to investigate lncRNA functions in cell biology.

### Cell transfection

Lentivirus-mediated short hairpin RNA for silencing *SNHG16* in cells and recombinant lentivirus for *SNHG16* overexpression were constructed by Genomeditech (Shanghai, China). Transfection was performed following the manufacturer's instructions. Lentiviruses were added to infect cells at a multiplicity of infection of 50:1.

### Statistical analysis

All data were expressed as the mean  $\pm$  SD. Statistical evaluation of two groups was conducted using Student's *t* test; a *P* value  $< 0.05$  was considered to indicate statistical significance.

## RESULTS

### Hypoxic culture facilitated hP-MSC proliferation

HIF-1 $\alpha$  is a critical regulator of cellular adaptation to the hypoxic microenvironment. When the expression of HIF-1 $\alpha$  protein under hypoxia was assessed by western blot, hypoxia induced the expression of HIF-1 $\alpha$  in hP-MSCs and stabilized its expression level during cell growth (Figure 1A). In addition, hP-MSCs cultured under hypoxia appeared to be relatively small, with a spindle-shaped morphology (Figure 1B). The cell counting kit-8 assay showed that hP-MSCs had higher proliferation potential ( $P < 0.0001$ ) when they were maintained under hypoxia (Figure 1C). Similarly, the colony-forming unit-fibroblast assay indicated that hypoxia enhanced the hP-MSC proliferation rate. Although the difference in the number of hP-MSC colonies between the hypoxia and normoxia groups was not significant ( $P = 0.249$ ), the colony size of the hypoxia group was larger with darker staining, indicating a higher number of cells (Figure 1D). c-MYC and proliferating cell nuclear antigen are molecules closely related to cell proliferation and can be adopted to determine the status of cell proliferation. As expected, hypoxia significantly increased the expression of c-MYC and proliferating cell nuclear antigen, indicating that cells proliferated more rapidly under hypoxia (Figure 1E).

### Hypoxia specifically altered the lncRNA and mRNA expression profiles of hP-MSCs

To further investigate the influence of hypoxia on hP-MSCs, whole-transcriptome sequencing was performed. First, six high-throughput sequenced transcriptomes were generated, containing over 650 million clean reads, among which three were from the normoxic group and three were from the hypoxic group. More than 96% of the raw reads were high-quality clean reads (Table 1). Ultimately, 10387 putative lncRNAs and 16041 mRNAs were identified. We further identified 289 differentially expressed lncRNAs (135 upregulated and 154 downregulated) and 240 differentially expressed mRNAs (156 upregulated and 84 downregulated) in the hypoxia group compared to normoxia group (Figure 2). Heatmap analysis clearly distinguished the hP-MSCs cultured under hypoxia from those cultured under normoxia. The top 20 differentially expressed lncRNAs and mRNAs are summarized in Tables 2 and 3.

### Differentially expressed mRNAs participated in cell proliferation function

The top ten enriched GO terms in biological process, molecular function, and cellular component were determined. For biological process, the differentially expressed mRNAs were related to regulation of cell growth (GO: 0001558), positive regulation of MAP kinase activity (GO: 0043406), and response to hypoxia (GO: 0001666) (Figure 3A). KEGG pathway enrichment analysis revealed several significantly enriched pathways, such as the HIF-1 signaling pathway (KEGG: hsa04066), Jak-STAT signaling pathway (KEGG: hsa04630), and Rap1 signaling pathway (KEGG: hsa04015) (Figure 3B). We further performed GO and KEGG analyses on the upregulated and downregulated genes separately. Upregulated genes were involved in regulation of cell growth (GO: 0001558) and regulation of cell proliferation (GO: 0042127) (Figure 3C) and were enriched in the HIF-1 signaling pathway (KEGG: hsa04066), Jak-STAT signaling pathway (KEGG: hsa04630), and AMPK signaling pathway (KEGG: hsa04152) (Figure 3D). Thus, hypoxia mainly affected cell functions, such as proliferation, by upregulating the expression of certain genes through several signaling pathways.

### SNHG16 was a potential promotor of hP-MSC proliferation ability

Hypoxia affected cell proliferation by regulating cell cycle progression as the percentages of S ( $P = 0.011$ ) and G2/M phase cells ( $P = 0.014$ ) were larger under the hypoxic condition (Figure 4A). At the same time, the PI3K/AKT pathway was activated under hypoxia (Figure 4B). The PI3K/AKT pathway is responsible for coordinating a diverse range of cell functions, including proliferation and survival. These findings suggest that hypoxia can activate the PI3K/AKT pathway and modulate the cell cycle.



**Table 1 Summary of the RNA sequencing data**

Summary	MSC_N1	MSC_N2	MSC_N3	MSC_H1	MSC_H2	MSC_H3
Raw reads (M)	103.22	113.01	117.09	114.00	117.77	106.81
Clean reads (M)	99.95	109.86	113.73	110.04	113.72	103.86
Clean reads rate (%)	96.83	97.21	97.13	96.53	96.56	97.24
Q30 (%)	94.32	94.63	94.62	94.22	94.18	94.78
GC (%)	46.71	45.65	45.35	46.18	44.99	45.92
Total mapped reads	320502139			324502629		
Uniquely mapped reads	308534583			312982527		

Q30 (%) represents the proportion of the data in which the quality values are > Q30 in the raw data; MSC\_N: Human placenta-derived mesenchymal stem cells cultured under normoxic condition; MSC\_H: Human placenta-derived mesenchymal stem cells cultured under hypoxic condition.

**Table 2 Summary of the top 20 differentially expressed long non-coding RNAs**

Upregulated lncRNA	Fold-change	Downregulated lncRNA	Fold-change
ENST00000480904	144.7046633	TCONS_00024987	558.6529515
ENST00000420168	88.58973499	TCONS_00022901	305.9141231
ENST00000447687	51.87793204	ENST00000580684	101.5147448
TCONS_00022897	43.87602707	XR_951092.3	96.15131381
NR_135828.1	43.01226344	TCONS_00040744	85.07807992
ENST00000652331	37.64067361	NR_151707.1	75.91477831
ENST00000615566	35.57471309	XR_924538.2	51.52143558
XR_001740831.1	30.29369126	ENST00000533146	47.14143364
XR_943245.2	29.4766044	NR_046472.1	45.2199291
ENST00000641463	29.12107436	ENST00000379848	34.42041339
ENST00000587838	28.77216197	XR_002957073.1	33.6627267
NR_027295.2	28.51478291	NR_138037.1	31.71645024
XR_001738493.2	26.08189868	ENST00000424751	31.28206241
ENST00000622955	24.98325918	ENST00000476224	29.12601955
ENST00000437589	23.11958922	NR_152515.1	28.62941025
NR_028397.1	22.10295425	ENST00000608741	25.94241325
NR_138259.1	21.16258012	XR_001740695.2	25.76946681
XR_947992.2	21.13351381	NR_102280.1	25.69150571
XR_930796.2	18.16731229	ENST00000513626	23.42886779
ENST00000542086	17.05330496	NR_132369.1	21.10943719

lncRNA: Long non-coding RNA.

To explore whether there are specific hypoxia-responsive lncRNAs that play a role in hypoxia-promoted cell proliferation, association analysis between lncRNAs and transcription factors and lncRNA-mRNA co-expression analysis were performed. *SNHG16* was related to the expression of *PIK3R5*, a gene encoding the regulatory subunit of the PI3K gamma complex (Figure 4C and D). *SNHG16* was also correlated with *FOSB*, a key transcription factor in the cell cycle (Figure 4E). Quantitative reverse transcription PCR analysis confirmed that hypoxia induced the expression of *SNHG16* ( $P = 0.003$ ), consistent with the results of RNA sequencing (Figure 4F). Thus, *SNHG16* is a potential promoter of hP-MSC proliferation under hypoxia.

Table 3 Summary of the top 20 differentially expressed messenger RNAs

Upregulated mRNA	Fold-change	Downregulated mRNA	Fold-change
<i>S100A1</i>	32.28708068	<i>MMP13</i>	32.7146796
<i>IL20</i>	31.01976446	<i>RASAL3</i>	25.22521191
<i>GUCY2D</i>	18.95647724	<i>ITGAM</i>	21.2376519
<i>PIK3R5</i>	16.27652505	<i>FGF14</i>	13.88641698
<i>KCNJ15</i>	9.485618545	<i>KCNS2</i>	9.402226087
<i>CA9</i>	9.092268285	<i>DCT</i>	7.332486828
<i>AK4</i>	8.577190243	<i>TMEM247</i>	6.224926429
<i>CD99</i>	7.853653254	<i>TNFRSF4</i>	5.89336975
<i>VSIG2</i>	6.545400641	<i>SOX7</i>	5.002461264
<i>CKMT2</i>	6.072884506	<i>TXNIP</i>	3.914446648
<i>FOLR1</i>	5.195878685	<i>NBPF6</i>	3.514713231
<i>C5orf46</i>	5.180793591	<i>PDF</i>	3.476083157
<i>PPFIA4</i>	4.979164209	<i>LHX4</i>	3.46662373
<i>TEC</i>	4.732591252	<i>CD14</i>	3.005111835
<i>INHBB</i>	4.597149928	<i>HHIPL2</i>	2.918560997
<i>GLDC</i>	4.140246682	<i>RASL11B</i>	2.770760137
<i>GPR146</i>	4.107008037	<i>LMO3</i>	2.767289903
<i>VASH2</i>	4.051761622	<i>MYH11</i>	2.728808288
<i>C4orf47</i>	3.969880654	<i>DIRAS2</i>	2.718905726
<i>SCHIP1</i>	3.945719906	<i>AJM1</i>	2.696612138

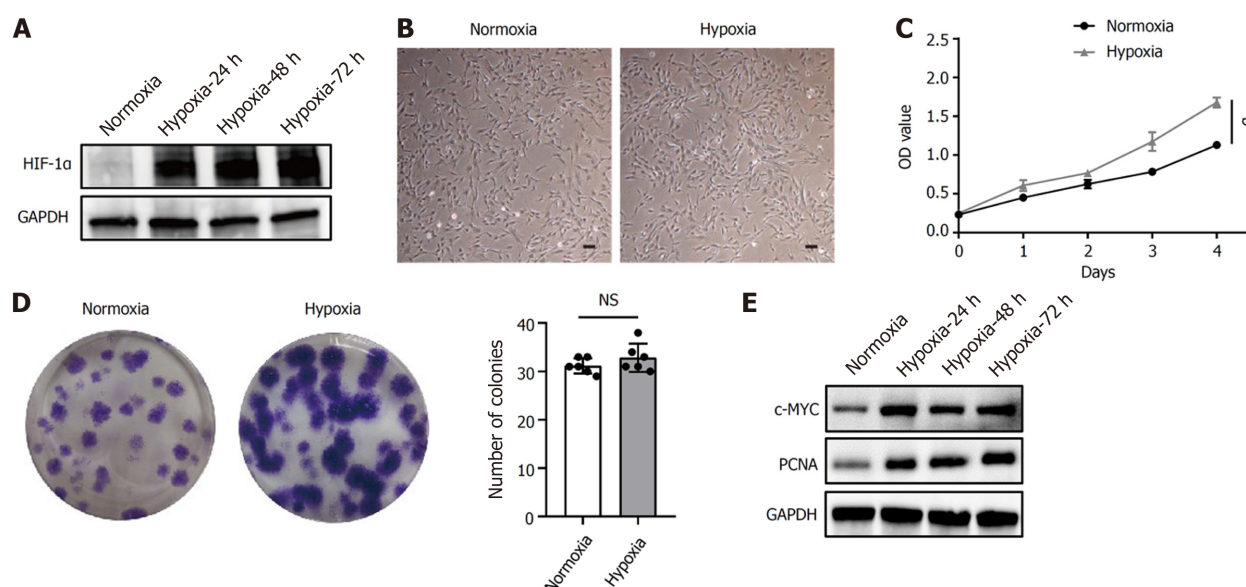
mRNA: Messenger RNA.

***SNHG16 promoted proliferation of hP-MSCs via the PI3K/AKT pathway***

To further confirm the biological function of *SNHG16* in hP-MSCs, short hairpin RNA was used to specifically knock down *SNHG16*, whereas lentivirus overexpressing *SNHG16* was used to increase *SNHG16* expression. By transfecting *SNHG16* short hairpin RNA, we found that sh-*SNHG16* significantly downregulated *SNHG16* expression ( $P < 0.0001$ ) by up to 80% (Figure 5A). The cell counting kit-8 assay then revealed that depletion of *SNHG16* could attenuate the proliferation ability of hP-MSCs under both normoxia ( $P = 0.0003$ ) and hypoxia ( $P = 0.0007$ ) (Figure 5B and Supplementary Figure 1). Moreover, *SNHG16* knockdown decreased the cell numbers in S phase ( $P = 0.022$ ) and increased the ratio of cells in the G0/G1 phase ( $P = 0.003$ ) (Figure 5C). Furthermore, western blot showed that knockdown of *SNHG16* downregulated the phosphorylation of AKT and the expression of several important cell cycle regulators, including CDK2, CDK4, CDK6, cyclin E1, and cyclin D1 (Figure 5D). Subsequently, we evaluated the effect of *SNHG16* overexpression on cell proliferation and cell cycle transition. Quantitative reverse transcription PCR indicated that *SNHG16* was upregulated approximately 8-fold ( $P = 0.0001$ ) when transfected with lentivirus overexpressing *SNHG16* (Figure 6A). Overexpression of *SNHG16* greatly enhanced the proliferative rate of hP-MSCs ( $P < 0.0001$ ) and caused a mild increase in the ratio of cells in the S ( $P = 0.027$ ) and G2/M phases ( $P = 0.003$ ) (Figure 6B and C). The expression of G1 to S phase transition-related genes in the *SNHG16* overexpression group increased along with the activation of the PI3K/AKT pathway (Figure 6D). Overall, these data demonstrated that *SNHG16* could facilitate the growth and cell cycle transition of hP-MSCs through activating the PI3K/AKT pathway.

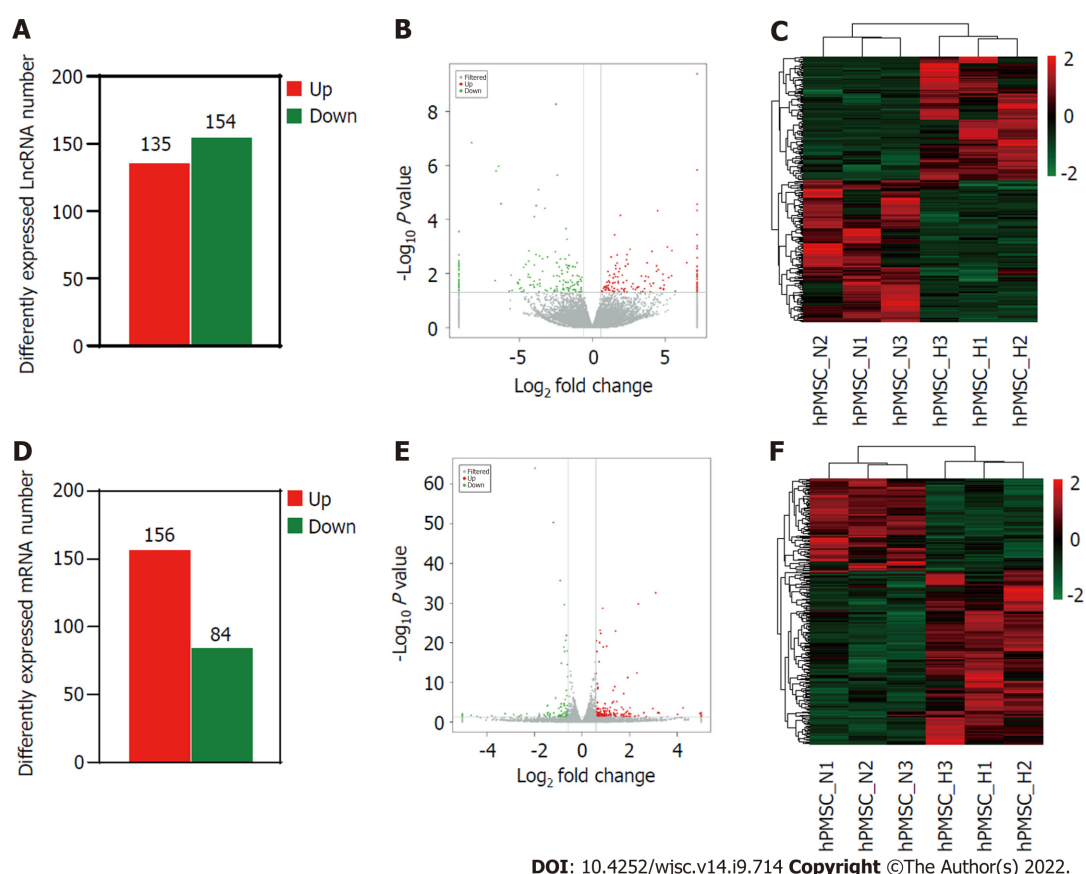
**DISCUSSION**

MSCs have great potential to cure a variety of diseases, as evidenced by the rapid growth in the number of published preclinical and clinical studies. However, MSCs are found in very small numbers in most adult tissues, such as bone marrow, placenta, adipose tissue, umbilical cord, amniotic fluid, and muscle



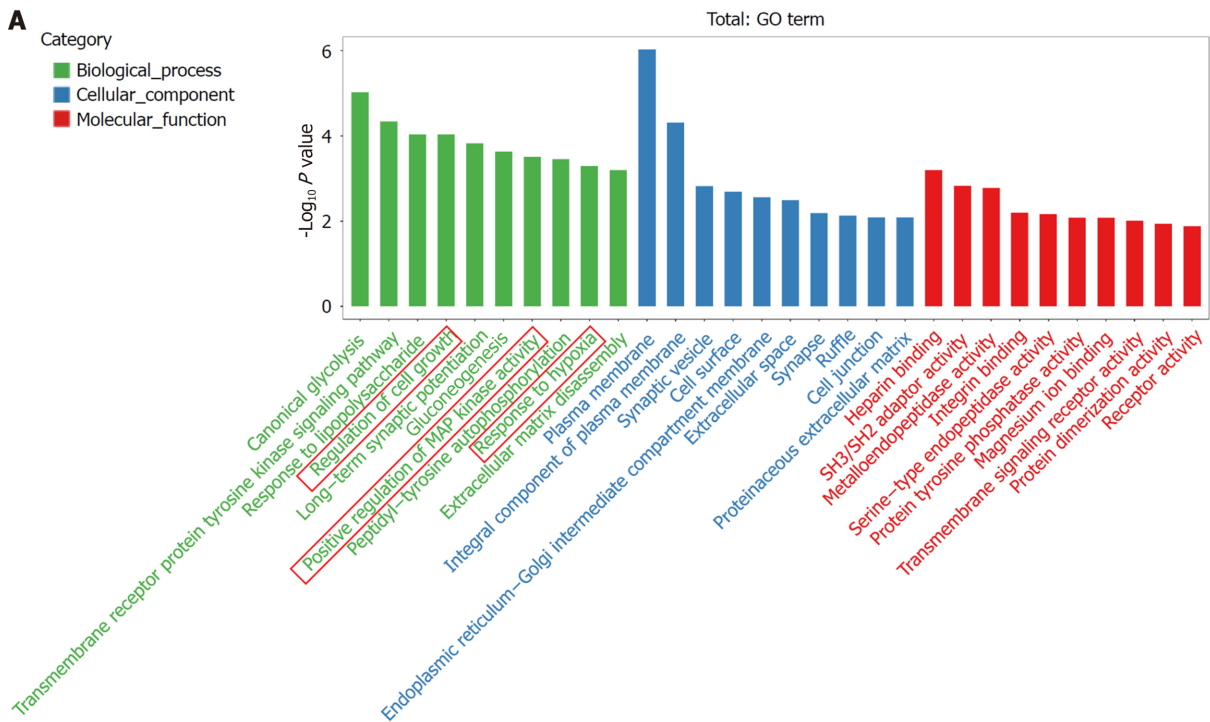
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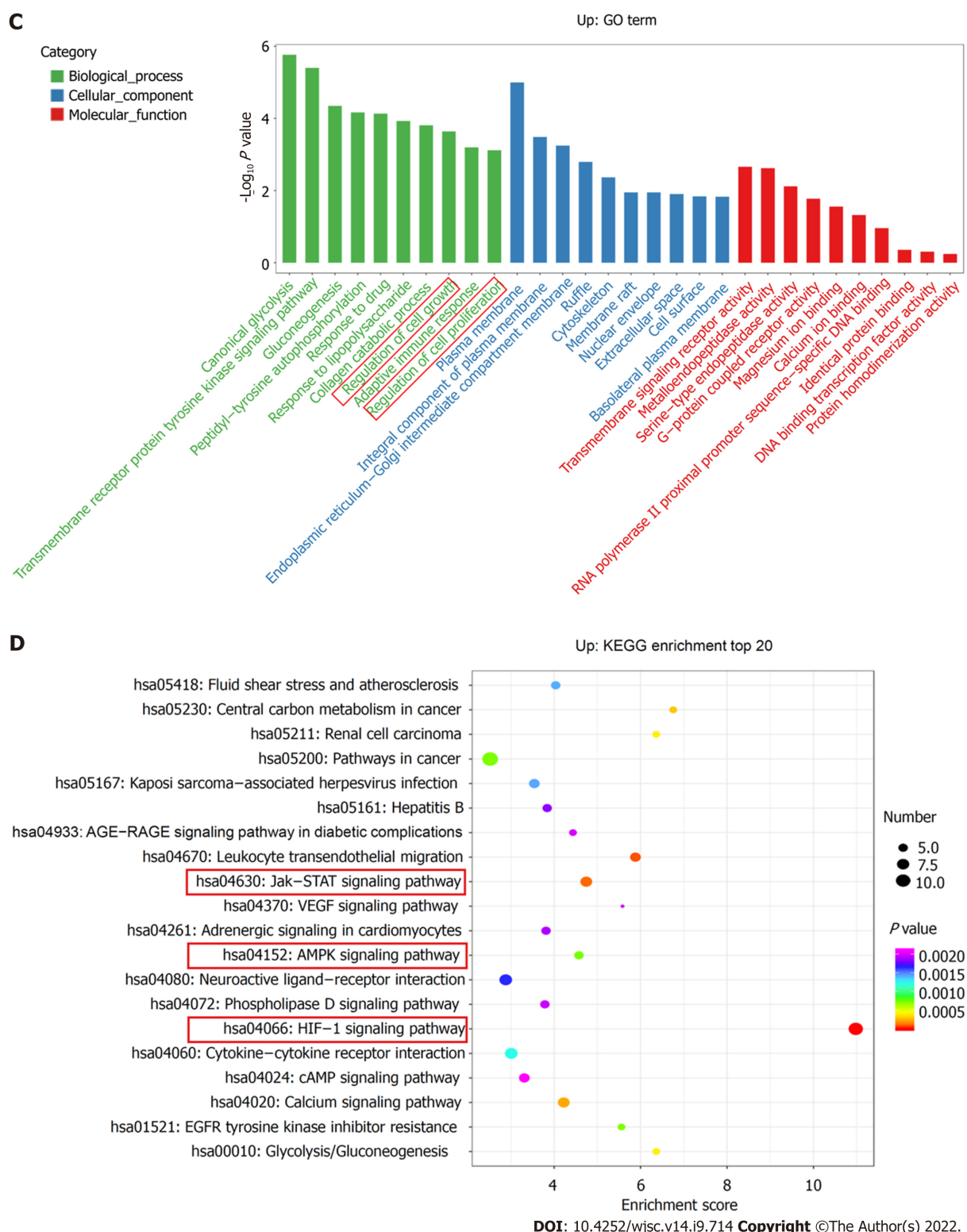
**Figure 1 Hypoxia facilitated human placenta-derived mesenchymal stem cell growth and proliferation.** A: Western blot analysis of hypoxia-inducible factor 1 $\alpha$  expression in human placenta-derived mesenchymal stem cells (hP-MSCs) under hypoxic culture for 24, 48, or 72 h; B: Morphology of the cultured hP-MSCs under hypoxia (scale bars, 100  $\mu$ m); C: Proliferation curves of hP-MSCs were established based on cumulative cell numbers at different incubation times (0, 1, 2, 3, and 4 d) under normoxia or hypoxia; D: Colony size and colony number of hP-MSCs under normoxic or hypoxic culture ( $n = 6$ ); E: The protein expression of c-MYC and proliferating cell nuclear antigen in hP-MSCs under hypoxic culture for 24, 48, or 72 h. Data are presented as means  $\pm$  SD.  $^aP < 0.05$ . NS: No significance; PCNA: Proliferating cell nuclear antigen; HIF-1 $\alpha$ : Hypoxia-inducible factor 1 $\alpha$ .



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**Figure 2 Long non-coding RNAs and messenger RNA expression profiles under hypoxia and normoxia.** A: Number of differentially expressed long non-coding (lnc)RNAs between hypoxia and normoxia; B: Volcano plot depicting differentially expressed lncRNAs between hypoxia and normoxia; C: Heatmap of all differentially expressed lncRNAs identified in hypoxia vs normoxia; D: Number of differentially expressed messenger (m)RNAs; E: Volcano plot of differentially expressed mRNAs; F: Heatmap showing hierarchical clustering of differentially expressed mRNAs.



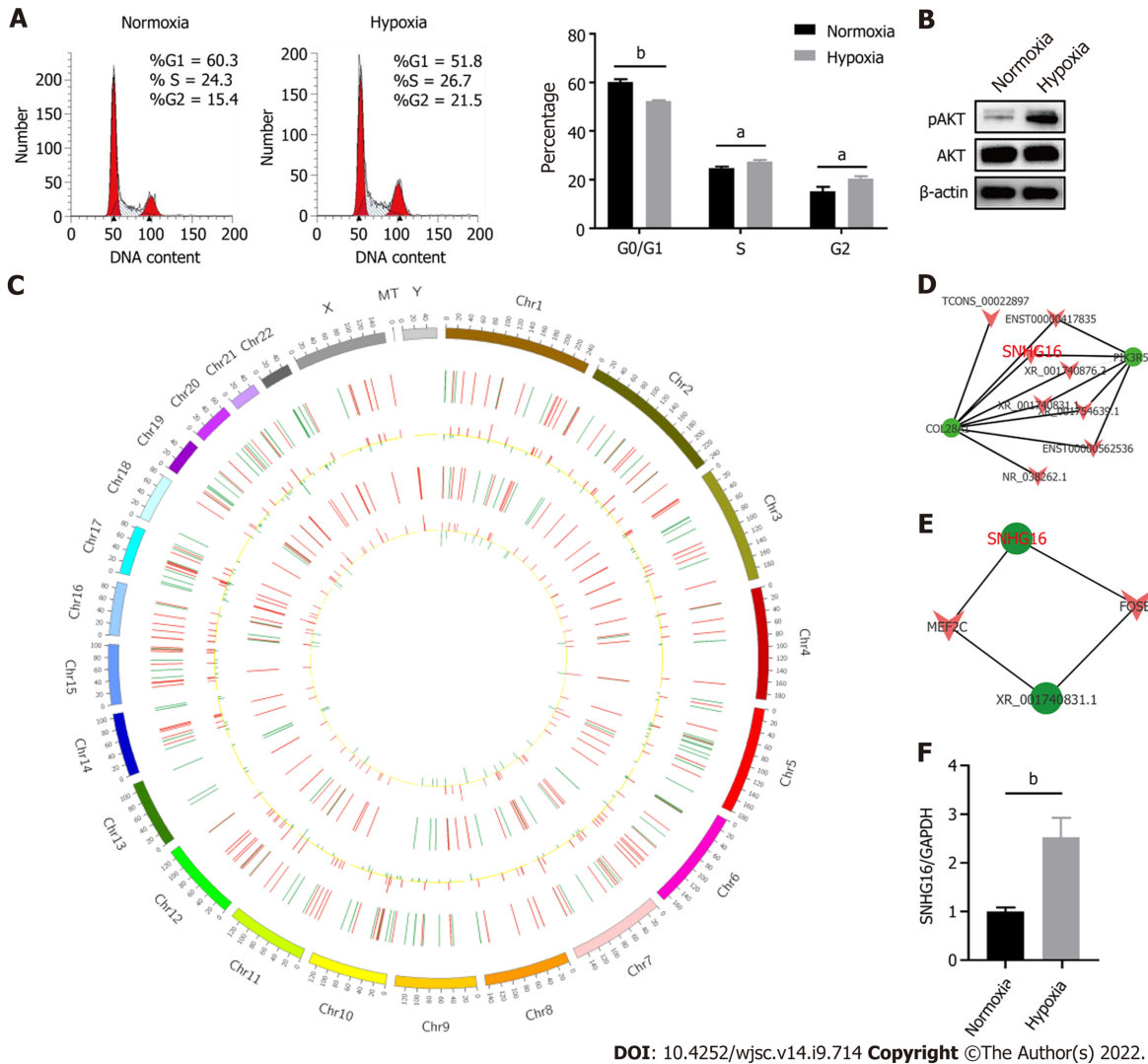


**Figure 3 Gene Ontology terms and Kyoto Encyclopedia of Genes and Genomes pathway analyses.** A: Enrichment of biological process, cellular component, and molecular function in all differentially expressed messenger RNAs (mRNAs); B: Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of all differentially expressed mRNAs; the top 20 are listed; C: Gene Ontology (GO) annotation and functional enrichment of upregulated mRNAs; D: KEGG pathway enrichment analysis of upregulated mRNAs.

[17,18]. To generate sufficient clinical therapeutic quantities, *in vitro* expansion is necessary[19]. Managing and modifying culture conditions during amplification of MSCs *in vitro* is critical for the manufacture of effective cell therapies, as these *in vitro* culture conditions affect the cell properties and cell behaviors after transplantation[20].

MSCs are widely located in the hypoxic microenvironment[21,22]. This physiological oxygen concentration is significantly lower than normoxic conditions typically used for MSC culture in the laboratory.





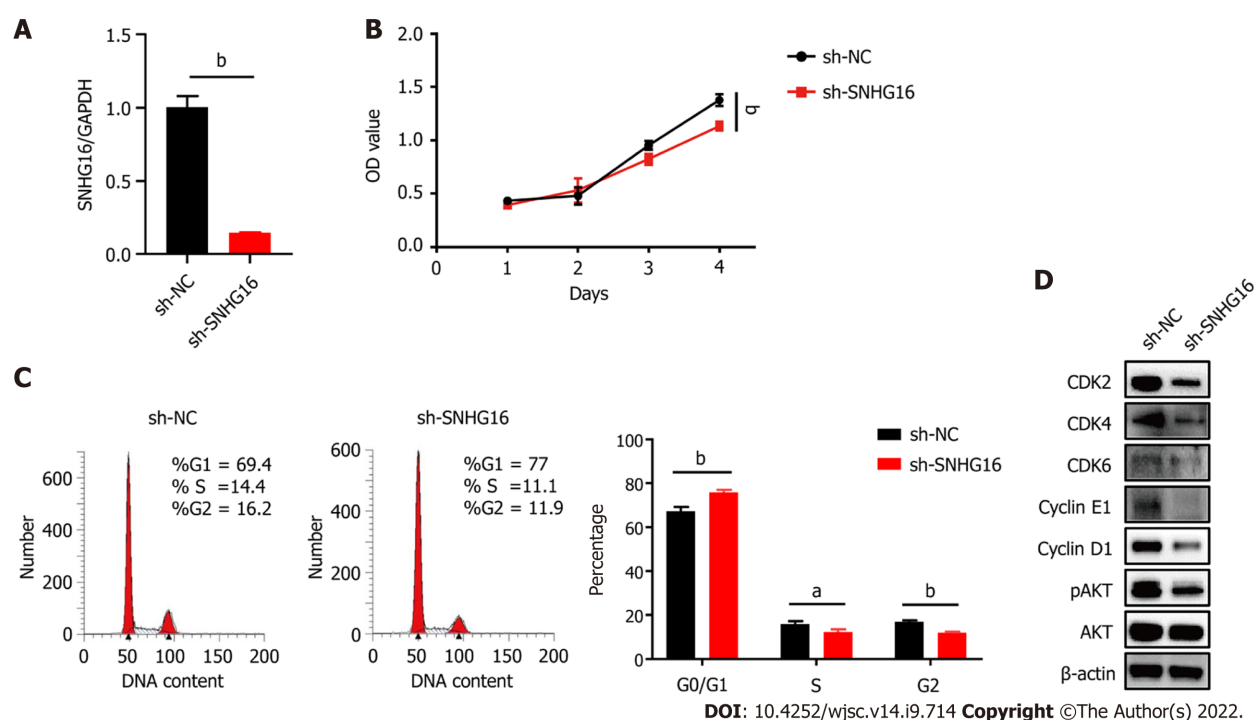
**Figure 4** SNHG16 was a potential promotor of human placenta-derived mesenchymal stem cell proliferation ability. A: Cell cycle analysis of human placenta-derived mesenchymal stem cells (hP-MSCs) under hypoxic culture via flow cytometry; B: Western blot analysis of AKT phosphorylation in hP-MSCs exposed to hypoxia; C: Circos plot of the long non-coding RNAs (lncRNAs)-messenger (m)RNA co-expression network. The outermost circle is the autosomal distribution. The second and third circles are the distribution of differentially expressed lncRNAs on chromosomes. The red line represents upregulation, and the green line represents downregulation. Higher bars indicate a greater number of differential genes in the interval. The fourth and fifth circles are the distribution of differentially expressed genes on chromosomes, with the same interpretation as lncRNA; D: Part of lncRNA-mRNA interaction network analysis visualized using the Cytoscape software; E: Part of the association analysis of differentially expressed lncRNAs and transcription factors; F: Effects of hypoxia on the expression of SNHG16 in hP-MSCs by quantitative reverse transcription polymerase chain reaction. Data are presented as means  $\pm$  standard deviation. <sup>b</sup> $P < 0.01$ .

Therefore, the application of physiological oxygen tension in stem cell research has attracted attention. Culturing MSCs under hypoxia has been consistently associated with increased cell proliferative rate, increased clonogenicity, decreased spontaneous differentiation, transcriptional alterations, and other cellular behaviors[11,23-25].

In the current study, we focused on the influence of hypoxia on hP-MSC proliferation ability. We found that hypoxic culture could facilitate hP-MSC proliferation, but enhanced clonogenicity under hypoxia was not observed in hP-MSCs. This finding provides a basis for exploring the underlying mechanism of the increased proliferation of hP-MSCs under hypoxic conditions.

Previous findings suggested that lncRNAs could exert regulatory function in MSC proliferation or differentiation. For example, Meng *et al*[26] revealed that *lincRNA-p21* promotes the migration and survival capabilities of mouse bone marrow-derived MSCs via the HIF-1 $\alpha$ /CXCR4 and CXCR7 axis under hypoxia[26]. In addition, *LINC01119* negatively regulates osteogenic differentiation of human bone marrow-derived MSCs, while the lncRNAs *LOC100126784* and *POM121L9P* improve the osteogenic differentiation of human bone marrow-derived MSCs[27,28]. However, the role of lncRNAs in hP-MSCs has rarely been reported.

Here, we employed RNA sequencing technology to obtain a comprehensive and systematic understanding of lncRNAs in hP-MSCs under hypoxia condition. A total of 289 lncRNAs (135 upregulated and 154 downregulated) and 240 mRNAs (156 upregulated and 84 downregulated) were differentially



**Figure 5 Knockdown of *SNHG16* attenuated the proliferation ability of human placenta-derived mesenchymal stem cells.** A: Quantitative reverse transcription polymerase chain reaction analysis of relative *SNHG16* expression after transfection of *SNHG16* short hairpin RNA (sh-*SNHG16*) and the corresponding controls (sh-NC) in human placenta-derived mesenchymal stem cells; B: Cell proliferation capacity evaluated by cell counting kit-8 assay; C: Cell cycle measured by flow cytometry; D: The G1 to S phase transition-related proteins and p-AKT detected by western blot analysis. Data are presented as means  $\pm$  standard deviation. <sup>b</sup> $P < 0.01$ .

expressed between the hypoxia and normoxia groups. Expression profiles of these differentially expressed genes were clustered hierarchically. GO and KEGG analyses suggested that the most enriched genes were positioned in the plasma membrane and related to regulation of cell growth and HIF-1 signaling pathway.

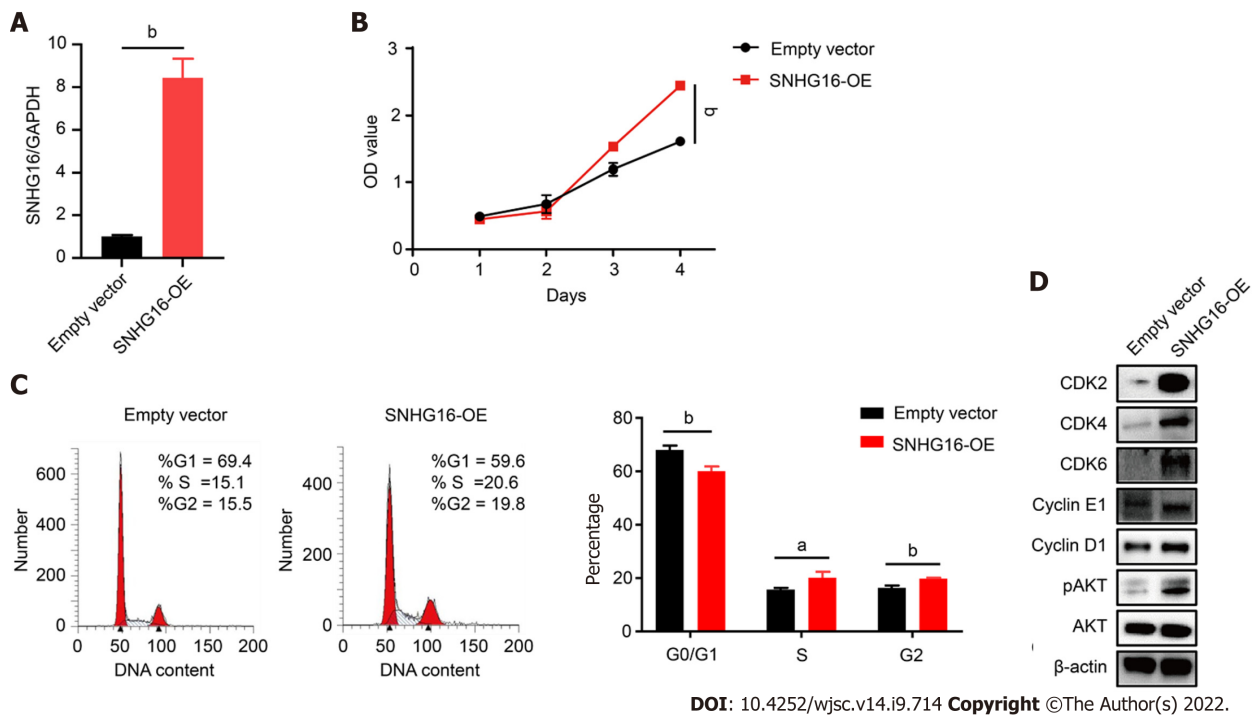
The results of the bioinformatic analysis were consistent with our experimental results. Combined with the individual analysis of upregulated genes, we found that hypoxia affected multiple cellular functions, mainly through upregulating the expression of certain genes. Moreover, hypoxia could mediate cell cycle progression and activate the PI3K/AKT pathway. Similarly, lncRNA-mRNA co-expression network analysis indicated that *SNHG16*, a hypoxia-responsive lncRNA, is associated with key genes in the cell cycle or PI3K/AKT pathway. Therefore, *SNHG16* was selected as a potential promoter of the hP-MSC proliferative rate under hypoxia.

*SNHG16* is a member of the SNHG family and is well-documented for its oncogenic properties in numerous types of malignancies[29]. *SNHG16* is reported to be involved in multiple cell biological functions, including cell cycle progression, proliferation, and migration[30-32]. In our study, we found that hypoxic culture could induce the expression of *SNHG16* in hP-MSCs. We further verified that *SNHG16* could promote cell cycle progression and cell proliferation of hP-MSCs by using knockdown and overexpression models. Moreover, we demonstrated that overexpression of *SNHG16* could increase the phosphorylation of AKT with a simultaneous elevation in the expression levels of G1 to S phase transition related proteins, including CDK2, CDK4, CDK6, cyclin E1, and cyclin D1.

However, how *SNHG16* becomes integrated in the PI3K/AKT signaling pathway in the study remains unknown. There are some related articles on the mechanism by which *SNHG16* regulates the AKT pathway in other models. For example, *SNHG16* could activate the PI3K/AKT pathway through *SNHG16*/*miR-338-3p*/*PLK4* axis in cisplatin-resistant neuroblastoma cells[33]. Moreover, *SNHG16* was found to facilitate proliferative diabetes-related abnormalities in cell proliferation through regulating *miR-7-5p*/*IRS1* to activate PI3K/AKT pathway in HG-stimulated hRMECs[31]. It can be seen that *SNHG16* mainly acts as a competing endogenous RNA to participate in the regulation of the PI3K/AKT signaling pathway. Our follow-up studies will take this as a starting point to elucidate the detailed mechanism of *SNHG16* regulation of the PI3K/AKT pathway.

## CONCLUSION

In this study, we have shown that hypoxia enhanced hP-MSCs proliferation ability and could



**Figure 6** *SNHG16* overexpression resulted in activation of the PI3K/AKT pathway and a significant enhancement in the proliferative rate of human placenta-derived mesenchymal stem cells. **A:** Quantitative reverse transcription polymerase chain reaction analysis of relative *SNHG16* expression after transfection of lentivirus overexpressing *SNHG16* (*SNHG16*-OE) and the corresponding empty vector in human placenta-derived mesenchymal stem cells; **B:** Cell proliferation after *SNHG16* overexpression was evaluated by cell counting kit-8 assay; **C:** Cell cycle distribution after *SNHG16* overexpression was evaluated by flow cytometry; **D:** The expression levels of CDK2, CDK4, CDK6, cyclin E1, cyclin D1, and phosphorylated AKT. Data are presented as the means  $\pm$  SD obtained from three separate experiments. <sup>b</sup> $P < 0.01$ .

specifically alter the lncRNA and mRNA expression profile. Furthermore, we identified a hypoxia-responsive lncRNA, *SNHG16*, which may serve as a regulator of promoting hP-MSCs proliferation under hypoxia. Mechanically, *SNHG16* was shown to activate the PI3K/AKT signaling pathway and upregulate the expression of key cell cycle regulators to induce cell cycle transition.

## ARTICLE HIGHLIGHTS

### Research background

As the role of hypoxia on mesenchymal stem cells (MSCs) is an emerging topic of MSCs biology, increasing studies are devoted to researching the regulation mechanisms of hypoxia on the biological functions of MSCs. Long non-coding RNAs (lncRNAs) and messenger RNAs (mRNAs) are reported to possess a critical role in regulating MSC biological characteristics. Nonetheless, the specific expression and co-expressed profiles of lncRNAs and mRNAs in human placenta-derived MSCs (hP-MSCs) under hypoxia and underlying mechanism of lncRNAs on hP-MSCs biology are still unknown.

### Research motivation

Although some studies have explored the effects of hypoxia on MSCs, the role of lncRNAs in them remains unclear.

### Research objectives

In this study, we aimed to reveal the specific expression profiles of lncRNAs in hP-MSCs under hypoxia and initially explored the possible mechanism of lncRNAs on hP-MSCs biology.

### Research methods

Here, we used a multigas incubator (92.5% N<sub>2</sub>, 5% CO<sub>2</sub> and 2.5% O<sub>2</sub>) to mimic a hypoxia condition and observed that hypoxic culture can significantly promote the proliferation potential of hP-MSCs. RNA sequencing technology was applied to identify the exact expression profiles of lncRNAs and mRNAs under hypoxia. After establishment of *SNHG16*-knockdown and *SNHG16*-overexpression hP-MSCs, the effect of *SNHG16* on proliferation capacity of hP-MSCs was analyzed *via* cell counting kit-8 and cell cycle analysis. Finally, the underlying mechanism was analyzed by western blot.

## Research results

We identified 289 differentially expressed lncRNAs and 240 differentially expressed mRNAs between hypoxia group and normoxia group. Among them, the lncRNA *SNHG16* was upregulated under hypoxia, which was also validated by reverse transcription polymerase chain reaction. *SNHG16* was confirmed to affect hP-MSCs proliferation rates by studying the *SNHG16* knockdown model. *SNHG16* overexpression could significantly enhance proliferation capacity of hP-MSCs, activate PI3K/AKT pathway, and upregulate the expression of cell cycle-related proteins.

## Research conclusions

Our results revealed the specific expression characteristics of lncRNAs and mRNAs in hypoxia-cultured hP-MSCs and identified that hypoxia-responsive lncRNA *SNHG16* can promote hP-MSC proliferation through the PI3K/AKT pathway.

## Research perspectives

This study may contribute to understanding the role of noncoding RNAs in MSC biology.

## FOOTNOTES

**Author contributions:** Feng XD contributed to the study design, experiments, data collection, and manuscript writing; Zhou JH, Chen JY, Feng B, Hu RT, and Wu J contributed to data collection and analysis; Pan QL, Yang JF and Yu J contributed to revising the manuscript; Cao HC contributed to design of the study, revision of the manuscript, and funding acquisition; All authors have read and approved the final manuscript.

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