Hypoxic preconditioning affects BMSCs proliferation and osteogenic differentiation by promoting paracrine release of HGF

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Introduction. Bone nonunion is one of the most common complications after long bone fracture fixation and may lead to dysfunction if not managed properly. Bone marrow mesenchymal stem cells (BMSCs) are the standard source of stem cells used in bone tissue engineering. Oxygen concentration is thought to have a crucial role in the proliferation, differentiation and self-renewal of BMSCs. The aim of this study was to investigate whether hypoxic preconditioning could affect the proliferation and osteogenic differentiation of BMSCs by promoting paracrine factors.

Methods. Based on bone marrow mesenchymal stem cells (BMSCs), hypoxia induction conditions were determined by cell activity and VEGF expression. Combined with proteomics sequencing to obtain differentially expressed paracrine factors in the supernatants of hypoxia-induced BMSCs cells and screen for key target genes (HGF). The supernatant of hypoxia-induced BMSCs serum-free medium was added to normally cultured BMSCs at a 1:1 ratio for 7d. Transfected si- HGF /NC and cell activity was detected by CCK8. Apoptosis was detected by flow cytometry. Transwell detects cell invasion. Cell scratch assay to detect cell migration.

Results. After hypoxic pretreatment we screened 204 up-regulated proteins and 369 down-regulated proteins. Analysis of the KEGG pathway revealed a significant role in the PI3K-Akt signaling pathway after hypoxic preconditioning. And by reviewing the literature, we found that HGF can participate in the expression of VEGF and has an important role in angiogenesis of bone marrow mesenchymal stem cells through the PI3K-Akt signaling pathway. From this, we chose HGF to study as a follow-up experiment. Hypoxia treatment

was able to significantly increase the expression of HGF in BMSCs. Hypoxia promotes cell

proliferation, migration, invasion and osteoblast differentiation and inhibits apoptosis. In contrast, interference with HGF inhibited cell proliferation, migration, invasion and osteoblast differentiation and promoted apoptosis.
Conclusion. Hypoxic preconditioning may affect BMSCs proliferation and osteogenic differentiation by promoting paracrine release of HGF.
Keywords. hypoxia; paracrine; HGF; bone marrow mesenchymal stem cells; osteogenic differentiation; cell proliferation

INTRODUCTION

Bone nonunion, also known as fracture nonunion, is one of the most serious complications of fractures, with a prevalence of 4% to 10%[1, 2]. The occurrence of bone nonunion is mostly related to poor local blood supply, inappropriate treatment methods, etc., and healing is not possible without further intervention [3]. Once bone nonunion occurs, the patient's function and quality of life will be seriously affected, long-term lower than the average level of the population, and even lower than patients with hypertension, diabetes, heart disease and stroke and other common diseases, becoming one of the most serious chronic diseases [4-6].

There are inconsistent reports on the role of hypoxia in bone marrow mesenchymal stem cells (BMSCs). It has been found that hypoxia reduces the ability of BMSCs to differentiate into osteoblasts, thereby impairing bone regeneration [7]. It has also been found that hypoxia promotes osteogenesis in human mesenchymal stromal cells in a hypoxia-inducible factor (HIF)-1-dependent manner [8]. Oxygen concentration is believed to have a crucial role in the proliferation, differentiation, and self-renewal of BMSCs, and hypoxic preconditioning of BMSCs significantly enhances their biological functions and activities, thereby improving the transplantation efficacy of BMSCs in therapeutic models of various diseases. It has been found that astragalus polysaccharides can promote the proliferation and osteogenic-induced differentiation

of BMSCs in a hypoxic environment [9]. It has been shown that cell culture

components derived from BMSCs after hypoxia treatment have increased vascularization, decreased cardiomyocyte apoptosis, and increased cardiac progenitor cell recruitment, and that hypoxia-pretreated BMSCs also ameliorate symptoms of spinal cord injury in rats by promoting their own cell survival and migration [10].

In this project, after the BMSCs were pre-treated with hypoxia, it was found that the expression levels of growth factors such as VEGF and TGF-β were significantly increased compared with those of the normal culture group, and after the obtained culture solution was added to the osteoblasts, it could significantly promote the proliferation of osteoblasts. It is hypothesized that the hypoxic secretome is enriched in molecules related to angiogenesis and tissue repair processes compared to the normoxic BMSC secretome. Therefore, we hypothesized that after pre-treating BMSCs with hypoxia, they can promote the migration, proliferation, and differentiation of BMSCs cells, as well as the migration and proliferation of osteoblasts through paracrine effects, thus providing more therapeutic ideas for their application in bone nonunion.

In this study, firstly, based on rat bone marrow mesenchymal stem cells (BMSCs), we screened for differentially expressed paracrine factors (hepatic growth factor, HGF) in the cellular supernatants of hypoxia-induced BMSCs by proteomic sequencing, and screened for genes related to VEGF. The regulatory effects of hypoxia-pretreated BMSCs on HGF and their effects on paracrine-related cytokines, cell biological activities and osteogenic differentiation were assessed by pre-transfection of si-HGF/NC.

MATERIALS AND METHODS

Cell Culture

Rat bone marrow mesenchymal stem cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. After removing the cells from the liquid

nitrogen tank, they were moved to a 37°C water bath to melt the cells rapidly. After

the cryopreservation solution is completely thawed, aspirate the cell suspension into a centrifuge tube, add 4 mL of complete medium, centrifuge at 400 g for 3 minutes, and discard the supernatant. Cells were resuspended in 1 mL of medium, transferred to culture flasks, 4 mL of complete medium was added, and incubated at 37°C in an incubator with 5% CO_2.

Cellular Hypoxia Model Construction

Cells of each group were collected, and the concentration of cell suspension was adjusted with complete medium, divided into 6-well plates, 5×10^5 cells/well, 2 mL per well, and placed in 37°C, 5% CO_2 incubator for 24 h. Cells were cultured under hypoxic conditions (2-8% oxygen) for 0, 24, 48, and 72 h. Cells from each group were collected for subsequent assays.

Cell Counting Kit-8（CCK8）

The cells were collected, the concentration of cell suspension was adjusted, and the cells were divided into 96-well plates with 3×10^3 cells/well, 100 μL per well, and incubated at 37°C in 5% CO_2 incubator overnight to make the cells adherent to the wall. Cells were treated according to different groupings and continued to be cultured for 7 d. Cell culture plates were removed and 10 μL of CCK8 solution (solarbio, Beijing, China) was added to each well and continued to be cultured for 4 h. The cells were then incubated for 4 h. The cell culture plates were then incubated for 4 h. Absorbance values of the wells were measured at 450 nm on the ELISA.

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA kits were used to detect the levels of VEGF and HGF in cell supernatants. All kits were purchased from Bioswamp (Wuhan, China). The experimental steps were carried out strictly according to the instructions of the kits.

Proteomic Screening of Differentially Expressed Paracrine Factors in the Supernatants of Hypoxia-induced BMSCs Cells

Mass spectrometry analysis using Label-free proteomics for non-labeled

quantification of differences in homologous proteins between multiple samples. Based

on the Raw files obtained from mass spectrometry, a search of the corresponding database was performed, and then protein identification was carried out based on the results of the database search, while peptide, protein, and parent ion mass tolerance distribution analysis was performed to assess the quality of the mass spectrometry data. Next, quantitative analysis of proteins was performed, including overall differential analysis of identified proteins and screening of differential proteins and clustering analysis of expression patterns. Finally, a series of differential protein functional analyses such as functional enrichment and interaction network analysis were performed for the screened differential proteins.

si-HGF Construction and Transfection

The interference plasmid HGF/NC was designed and constructed based on the sequence of the gene HGF screened by sequencing. Transfection into BMSCs was performed 24 h before transfection by inoculating 3×10^5 cells in 2 mL complete medium, seeded in 6-well plates, and the cell fusion was 70 % at the time of transfection. Dilute 4 μg of plasmid DNA with 250 μL of Opti-MEM and gently blow 5 times to mix. Dilute 10 μL of Lipofectamine 2000 with 250 μL of Opti-MEM, gently blow 5 times to mix, and let stand for 5 min at room temperature. Then mix the liquid, gently blow 5 times to mix, and let it stand for 20 min at room temperature. Add 500 μL of the complex to the wells of a culture plate containing cells and swapped with 1.5 mL of fresh medium, and gently rock the cell culture plate back and forth. The cell plates were placed in a 37°C, 5% CO_2 incubator, transfected for 4 hours and then replaced with fresh medium and incubated for 48 h. The transfection efficiency was verified by detecting the expression of gene A by qRT-PCR.

qRT-PCR

Cells in each group were collected, total RNA of each group was extracted using TRIzol reagent and the concentration was detected, then reverse transcribed into cDNA according to the TaKaRa kit instructions, and amplified for detection. Reaction

conditions: pre-denaturation at 95°C for 30s, denaturation at 95°C for 5s,

annealing/extension at 60°C for 30s, a total of 40 cycles. The relative expression of target gene was calculated by $2^{-\Delta\Delta CT}$. The primer sequences were as follows in Table 1.

Flow Cytometry

Cells were collected from each group and 1×10^6 cells resuspended in culture medium were taken at 400 g and centrifuged at 4°C for 5 min. Add 1mL of pre-cooled PBS, gently blow to mix the cells, and centrifuge at 400 g for 5min at 4°C. Resuspend the cells in 200 μL of PBS. Add 10 μL of Annexin V-FITC and 10 μL of PI (BD, US), mix gently, and incubate for 30 min at 4°C away from light. add 300 μL of PBS, followed by flow-through detection. Analysis was performed using NovoExpress analysis software.

Transwell

5×10^4 cells were inoculated in the upper layer of the Transwell, and 100 μL of DMEM medium without fetal bovine serum was added, along with 400 μL of DMEM medium containing 100 ml/L fetal bovine serum based on the lower layer of the chamber. Cells that had not migrated through the membrane remaining in the upper layer of the chambers were swabbed off after 48 h of routine incubation, and then the chambers were fixed in paraformaldehyde for 15 min, stained with crystal violet for 30 min, and placed upside down at room temperature to air-dry overnight. Transwell invasion assay requires Matrigel adhesive to be spread into the upper layer of the chambers before inoculation of cells, and the rest of the steps are the same as the migration assay. Finally, 10 random visual field counts were observed under the microscope. Three replicate wells were set up and each experiment was repeated three times.

Western-blot

RIPA buffer lysed cells were assayed for protein concentration using BCA. Protein

electrophoresis was performed by adding 25g of protein samples per well to

SDS-PAGE gel, electrotransferred to PVDF membrane, and closed with 50g/L skimmed milk for 1h. Add primary antibody HGF(PAB30836, 1:1000), VEGF(PAB43767, 1:1000), TGF-β(PAB45878, 1:1000), BMP-2(PAB30060, 1:1000), FGF2(PAB30064, 1:1000), MMP-2(PAB34434, 1:1000), MMP-9(PAB30102, 1:1000), GAPDH(PAB36269, 1:1000), and place in 4°C refrigerator overnight. The secondary antibody was added and incubated at room temperature for 2 h. A DNR bioimaging system was applied for luminescence and image acquisition.

Statistical Analysis

The experimental results of this study were statistically analyzed using SPSS 20.0 software. The variables of interest in the study conformed to normal distribution and the data for the measures are expressed as mean ± standard deviation (x ± s). T-tests were used for comparisons between the two groups, and one-way ANOVA and Dunnett 's post hoc tests were used to analyze multiple comparisons, with the variances within each experimental group not being significantly different from each other. Bilateral $P<0.05$ was considered a statistically significant difference.

RESULTS

Hypoxia Induces Differential mRNA Expression in BMSCs and Screens for Key Target Genes

We found that hypoxic incubation for 24, 48, and 72 h increased the proliferative capacity and VEGF levels of cells compared to 0 h (Figure 1A-B). The best results were obtained at 48h of induction. Therefore, we chose to hypoxia-induce BMSCs cells for 48h for subsequent experiments. We then screened for differentially expressed paracrine factors in the supernatants of hypoxia-induced BMSCs cells by proteomics. We screened 204 up-regulated proteins and 369 down-regulated proteins (Figure 1C). Its volcano diagram is shown in Figure 1D.

Significant Enrichment and KEGG Analysis of Differential Protein GO Functions

GO analysis showed that the functional annotations indicated that the differential proteins mainly functioned in the direction of molecular functions, cellular components and biological processes. Among the biological processes are mainly in extracellular matrix organization, proteolysis and cell adhesion. Cellular components are mainly represented in the outer cell space, the plasma membrane, and components of the membrane. The molecular functions are mainly manifested in the MOLECULAR ADAPTER ACTIVITY and STRUCTURAL MICROCULE ACTIVITY (Fig. 2). Differential proteins were significantly enriched for the KEGG pathway showing a significant role on the PI3K-Akt signaling pathway after hypoxic preconditioning (Figure 3A). Then, we found that a number of proteins (F1LSB2, G3V6A0, COL6a1, HGF and Hsp90aa1, among others) are associated with the PI3K-Akt signaling pathway. Among them, COL6a1, HGF and Hsp90aa1 were able to participate in VEGF expression [11-13]. The expression of COL6a1, HGF and Hsp90aa1 was found to be significantly increased in the cells of the hypoxia group compared with the normoxia group by ELISA (Figure 3B). Upon review of the literature, HGF was found to have an important role in angiogenesis of bone marrow mesenchymal stem cells through the PI3K-Akt signaling pathway [14]. Therefore, we first chose HGF for the follow-up study. to investigate whether hypoxic pretreatment of BMSCs affected the proliferation and osteogenic differentiation of BMSCs cells by mediating the expression of HGF.

Hypoxic Preconditioning Affects BMSCs Cell Proliferation and Apoptosis by Promoting Paracrine HGF Release

Compared with the NC group, the expression of HGF was significantly reduced in all three HGF-interfering sequences we constructed, with si-HGF-1 showing the best interference efficiency (Figure 4A). Therefore, we chose si-HGF-1 for subsequent experiments. The level of VEGF was significantly higher in the cells of the si-HGF group compared with the NC group (Figure 4B). Conditioned medium induction

conditions were screened by CCK8 experiments. It was found that the proliferative

capacity of the cells in the 1:0, 1:1, 1:2, and 1:4 ratio groups was increased compared to the 0:1 group. The 1:1 ratio was the most effective, and the proliferative capacity of the cells was significantly increased at both 5 and 7 d of culture (Fig. 4C). Therefore, we chose a 1:1 ratio to be added to the complete medium culture of BMSCs cells cultured for 7 d for subsequent experiments. Compared with the Normoxic group, the proliferation ability of cells in the Hypoxia group was significantly increased and the apoptosis rate was significantly decreased. Compared with the NC+Hypoxia group, cells in the si-HGF+Hypoxia group showed significantly reduced proliferation and increased apoptosis (Figure 4D-F). Meanwhile, the migration and invasion of cells were significantly increased in the Hypoxia group compared with the Normoxic group. The migration and invasion of cells were significantly reduced in the si-HGF+Hypoxia group compared with the NC+Hypoxia group (Figure 5A-C). This result indicates that hypoxia promotes cell proliferation, migration and invasion, and inhibits apoptosis, whereas interference with HGF inhibited cell proliferation, migration and invasion, and promoted apoptosis.

Hypoxic Preconditioning Affects Osteogenic Differentiation of BMSCs Cells by Promoting Paracrine Release of HGF

Protein expression of HGF, VEGF, TGF-β, BMP-2, FGF, MMP-2, and MMP-9 was significantly upregulated in cells of the Hypoxia group compared with the Normoxic group. Protein expression of HGF, VEGF, TGF-β, BMP-2, FGF, MMP-2, and MMP-9 was significantly downregulated in cells of the si-HGF+Hypoxia group compared with the NC+Hypoxia group (Figure 6A). In addition, the mRNA expression levels of HGF, Runx2, ALP, OSX and OPN were significantly increased in the cells of the Hypoxia group compared with the Normoxic group. The mRNA expression levels of HGF, Runx2, ALP, OSX, and OPN were significantly reduced in the cells of the si-HGF+Hypoxia group compared with the NC+Hypoxia group (Figure 6B). This result again demonstrates that hypoxia promotes cell migration and

invasion, and promotes osteogenic differentiation of cells. and interference with HGF both reversed these results.

DISCUSSION

Nonunion is a common clinical problem, and conservative treatment is less effective[15]. BMSCs have the ability of self-renewal and multidirectional differentiation, and can maintain the number of their own cells through proliferation and differentiation, and can be differentiated into osteoblasts, neuronal cells, adipocytes, glial cells, endothelial cells, etc. under appropriate conditions[16, 17], fulfills the need for restoration of areas of osseous nonunion. We have found previously that hypoxic preconditioning of BMSCs significantly promotes the proliferation of osteoblasts. However, the mechanism of action on osteoblasts after hypoxic preconditioning of BMSCs has not been clarified. In the present study, we found that we screened 204 up-regulated proteins and 369 down-regulated proteins after hypoxic preconditioning, in which the expression of HGF was significantly increased. And hypoxic preconditioning affected BMSCs proliferation, migration, invasion and osteoblast differentiation and inhibited apoptosis by promoting paracrine release of HGF.

Currently, there are inconsistent reports on the role of hypoxia in BMSCs. Shen et al. [18] found that hypoxic preconditioning could optimize the therapeutic effects of BMSC-derived exosomes, as evidenced by the promotion of articular chondrocyte proliferation, migration, anabolism, and anti-inflammatory effects. Zhao et al. [19]showed that hypoxic preconditioning promoted BMSC viability, increased hypoxia-inducible factor 1 α (HIF-1α), vascular endothelial growth factor (VEGF), alkaline phosphatase (ALP) expression, calcium deposition, and enhanced the formation of vessel-like structures. In contrast, Zhang et al. found that exosomes derived from hypoxic BMSC were able to be taken up by neighboring cancer cells

and promoted cancer cell invasion and EMT [20]. In this study, we first analyzed the

differential proteins of hypoxia pretreated BMSCs and found 204 up-regulated proteins and 369 down-regulated proteins, and found that hypoxia pretreated played a significant role in PI3K-Akt signaling pathway by KEGG pathway analysis. And by reviewing the literature, we found that HGF can participate in the expression of VEGF and has an important role in angiogenesis of bone marrow mesenchymal stem cells through the PI3K-Akt signaling pathway. It has been found that BMSCs can not only directly migrate and differentiate into osteoblasts, but also regulate vascularization, inhibit osteoblastic fibrosis, resist apoptosis and regulate osteogenic differentiation in the damaged area by secreting various bioactive molecules and extracellular vesicles [21].

HGF is a multi effector cytokine with important anti-fibrotic and anti-apoptotic effects and is involved in liver regenerative processes [22]. It has been found that overexpression of HGF by BMSCs significantly enhanced hepatocyte regeneration, inhibited apoptosis, and reversed the progression of liver fibrosis [23]. However, exogenous HGF is highly unstable and does not maintain consistently high levels in the circulation even with multiple repeated injections [24]. In the present study, we found that hypoxia preconditioned BMSCs released HGF by promoting paracrine secretion. Hypoxia significantly increased the expression of HGF in BMSCs and promoted cell proliferation, migration, invasion and osteogenic differentiation, and inhibited apoptosis. And all of these results were reversed after interfering with HGF. Therefore, hypoxic preconditioning may affect the proliferation and osteogenic differentiation of BMSCs by promoting paracrine release of HGF.

CONCLUSION

In summary, based on the results of this study, we concluded that hypoxic preconditioning may affect BMSCs proliferation and osteogenic differentiation by promoting paracrine release of HGF. This provides more theoretical basis for the application of hypoxic preconditioned BMSCs in the treatment of bone nonunions.

However, this study still has certain limitations, and further research is needed on the pathway mechanism of HGF released by hypoxic preconditioning on bone nonunion.

AVAILABILITY OF DATA AND MATERIALS

All data from this study can be requested directly from the corresponding author upon reasonable request.

CONFLICTS OF INTEREST

All authors declare that they have no conflict of interest.

FUNDING

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Table 1 Primer sequence table.

Primer name	sequences	Size(bp)
Runx2-F	GGACGAGGCAAGAGTTTCAC	195
Runx2-R	ACTGGGATGAGGAATGCG	
HGF-F	TTATGGGGAATGAGAAATGC	217
HGF-R	CGAACAAAAATACCAGGACG	
ALP-F	AAGCAGCATCTTACCAGTT	114
ALP-R	CAGCCTCTAGGTTGATTTTA	
OSX-F	CCATTGCCAGTAATCTTCGT	169
OSX-R	TTTCCCAGGGCTGTTGAG	
OPN-F	GTTTGCTTTTGCCTGTTCG	101
OPN-R	GCATCTGAGTGTTTGCTGTAA	
HGF-F	TTATGGGGAATGAGAAATGC	217
HGF-R	CGAACAAAAATACCAGGACG	
GAPDH-F	CAAGTTCAACGGCACAG	138
GAPDH-R	CCAGTAGACTCCACGACAT	

FIGURE LEGENDS

Figure 1 Differential mRNA expression and screening of key target genes based on BMSCs in good culture condition, cultured under low oxygen conditions (2-8% oxygen) for 0, 24, 48 and 72 h. A: CCK8 assay to detect the activity of each group of cells.B: ELISA to detect the expression of VEGF in the supernatant of the cells.C: Statistical graph of the number of differential proteins.The X-axis indicates the number of differential proteins, and the Y-axis indicates the number of group comparison conditions, red indicates the number of up-regulated differential proteins, and blue indicates the number of down-regulated differential proteins.D: Differential protein volcano plot. x-axis indicates the multiplicity of differences of differential proteins (log2 value), y-axis indicates the pvalue (-log10 value), and the grey color represents the proteins whose differences are not significant, and the red color represents the up-regulated proteins, and the blue color represents the down-regulated proteins.

Figure 2 According to the GO enrichment results, the top 20 enrichment function entries are taken by default to draw a bubble chart according to the P-value of the enrichment analysis sorted from smallest to largest.

Figure 3 Differential protein KEGG analysis and validation. a: KEGG enrichment bar graph. x-axis denotes Rich Factor, y-axis denotes enriched KEGG entries. b: Expression of COL6a1, HGF, and Hsp90aa1 was detected in cell supernatants by ELISA.

Figure 4 Hypoxia pretreatment affects the proliferation and apoptosis of BMSCs cells by promoting paracrine release of HGF. A: HGF expression was detected by qRT-PCR to validate the transfection efficiency. B: The level of VEGF in the cells

was detected by ELISA. C: The supernatant of serum-free medium of BMSCs treated

with hypoxia for 48 h was collected, and added to the completely medium-cultured BMSCs cells, and the cell activity was detected by CCK8 after 1, 3, 5, and 7 d of culture. D: Detection of cell activity in each group by CCK8.E-F: Detection of cell apoptosis in each group by flow cytometry.E: Detection of apoptosis in each group by flow cytometry.

Figure 5 Hypoxic preconditioning affects the migration and invasion of BMSCs cells by promoting paracrine release of HGF. a: cell migration was detected by cell scratch assay. b-c: cell invasion was detected by Transwell.

Figure 6 Hypoxic preconditioning affects osteogenic differentiation of BMSCs cells by promoting paracrine release of HGF. a: Changes in protein expression of HGF, VEGF, TGF-β, BMP-2, FGF, MMP-2, and MMP-9 were detected by western-blot in the cells. b: Changes in mRNA expression levels of runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), Osterix protein (OSX) and osteoblastic protein (OPN) mRNA expression levels in the cells of each group.

Figure 1

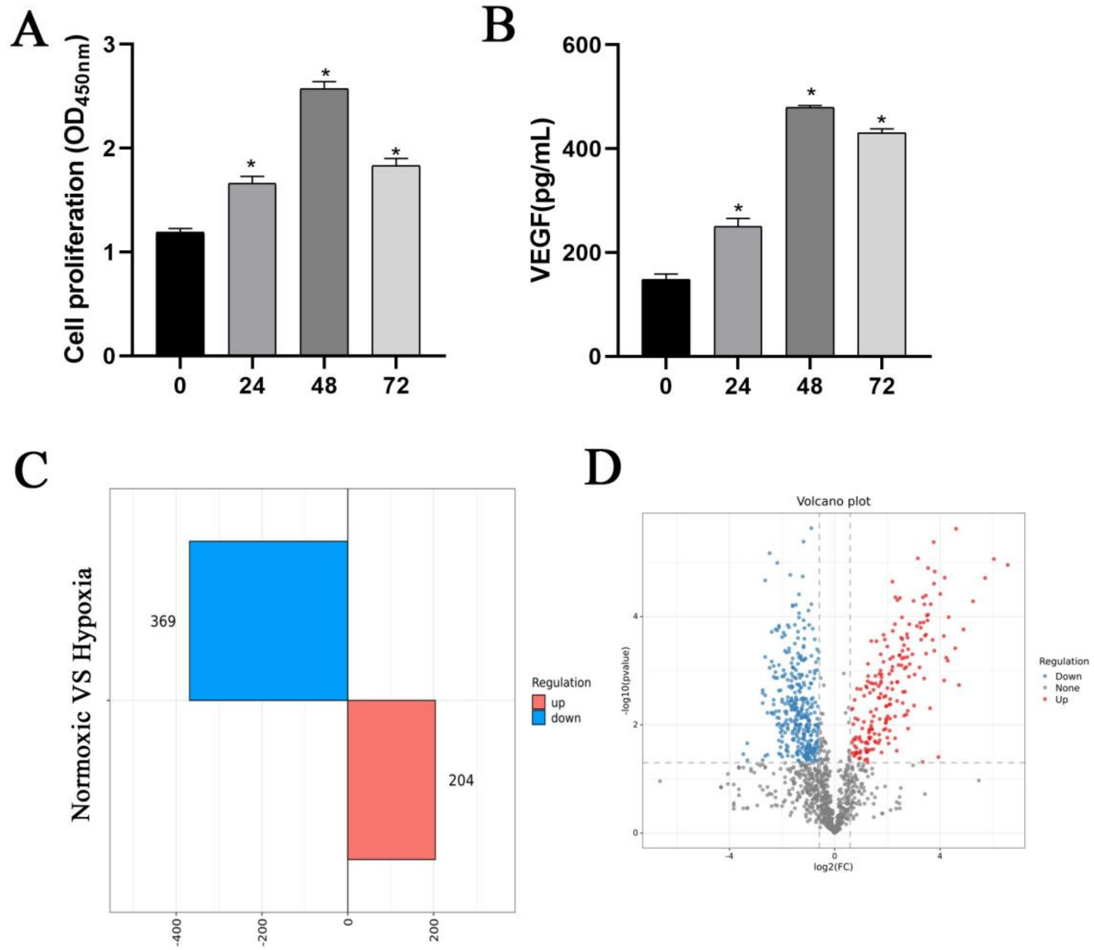

Figure 2

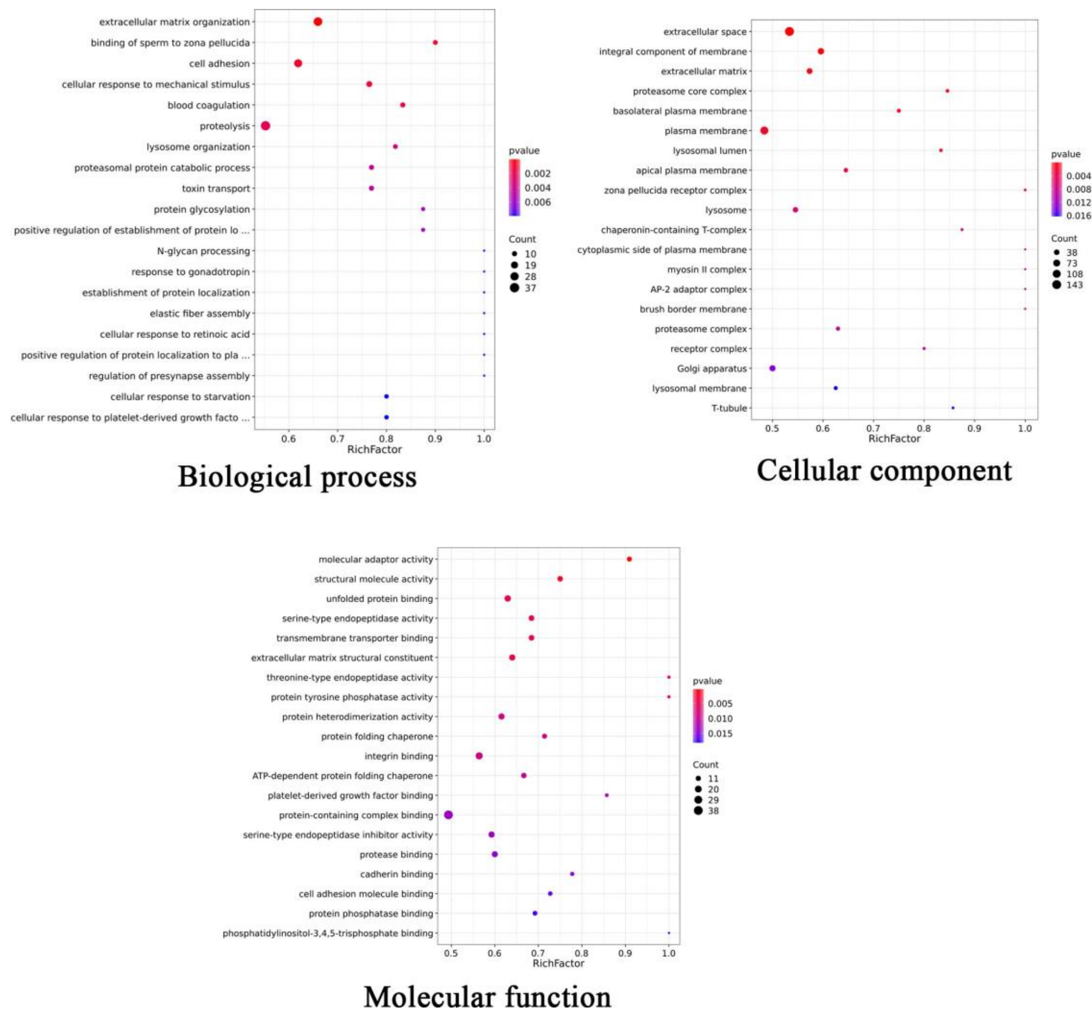

Figure 3

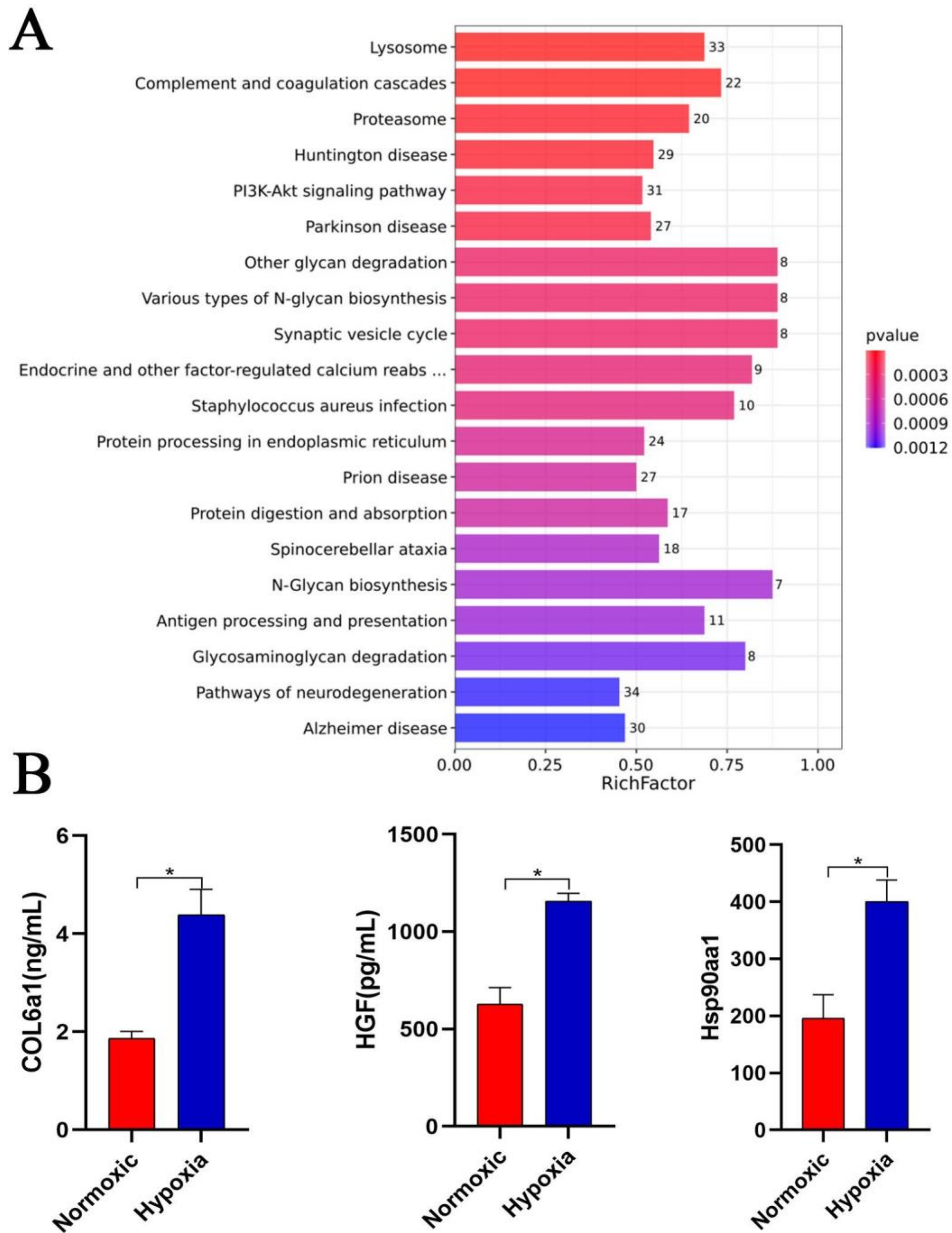

Figure 4

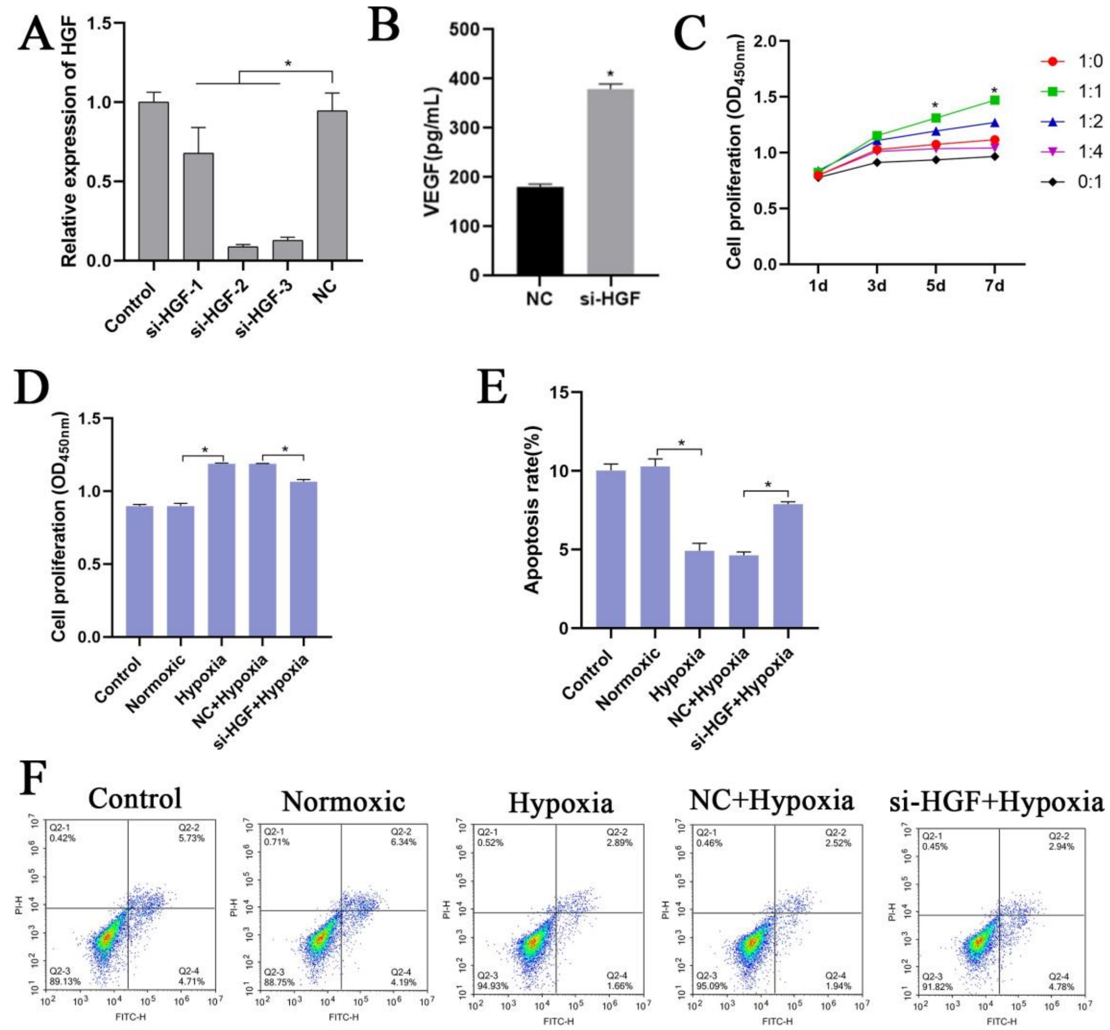

Figure 5

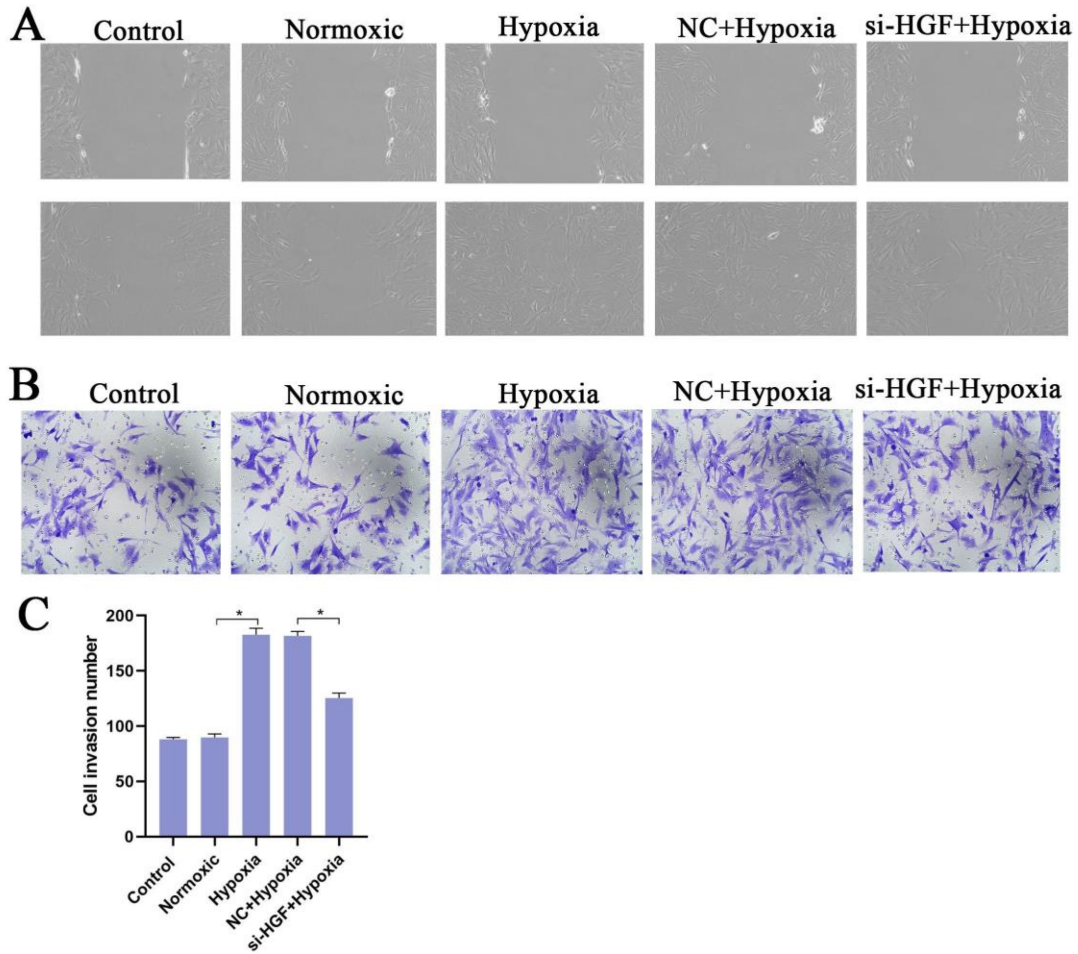

Figure 6