

# A genome-wide analysis of the gene expression profiles and alternative splicing events during the hypoxia-regulated osteogenic differentiation of human cartilage endplate-derived stem cells

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**Abstract.** It has been hypothesized that intervertebral disc degeneration is initiated by degeneration of the cartilage endplate (CEP), which is characterized by cartilage ossification. CEP-derived stem cells (CESCs), with the potential for chondro-osteogenic differentiation, may be responsible for the balance between chondrification and ossification in the CEP. The CEP remains in an avascular and hypoxic microenvironment; the present study observed that hypoxia was able to markedly inhibit the osteogenic differentiation of CESCs. This tissue-specific CESC differentiation in response to a hypoxic microenvironment was physiologically important for the prevention of ossification in the CEP. In order to study the hypoxia-regulated mechanisms underlying osteogenic differentiation of CESCs, a Human Transcriptome Array 2.0 was used to detect differentially expressed genes (DEGs) and alternatively spliced genes (ASGs) during the osteogenic differentiation of CESCs under hypoxia, compared with those induced under normoxia. High-throughput analysis of DEGs and ASGs demonstrated that genes in the complement pathway were enriched, which may be a potential mechanism underlying hypoxia inhibition of CESCs osteogenesis. The results of the present study may provide a basis for future

mechanistic studies regarding gene expression levels and alternative splicing events during the hypoxia-regulated inhibition of osteogenesis, which may be helpful in identifying targets for CEP degeneration therapy.

## Introduction

Lower back pain (LBP) is a common disorder that induces activity limitation (1). Degenerative disc disease (DDD) is a common cause of LBP (2). Numerous factors have been reported to be associated with the pathogenesis of DDD, including mechanical stress (3), cellular senescence (4) and extracellular matrix degradation (5). Among these mechanisms, the decreased transport of nutrition and waste products appears to be the most important (6,7). The intervertebral disc (IVD) is the largest avascular tissue in the human body, and the metabolic exchange of a mature IVD is largely dependent on diffusion through the cartilage endplate (CEP) (8). The CEP is a thin horizontal layer of hyaline cartilage that separates adjacent vertebrae from the IVD. The blood vessels in the vertebral bones do not invade the discs, ending at the interface between the IVD and the vertebral body (9). As the most important channel for metabolic exchange, the degeneration of the CEP is hypothesized to be predominantly responsible for the initiation of DDD (10).

CEP degeneration is characterized by ossification, rather than chondrification (11). Chondrification is important for the physiological functioning of the CEP, whereas ossification is harmful to the ability of the cartilage to resist compressive forces and disrupts the transport properties of the CEP (12). However, the mechanisms underlying ossification in the CEP remain unclear.

A previous study demonstrated the presence of CEP-derived stem cells (CESCs), which may undergo osteogenic and chondrogenic differentiation (13). The differentiation characteristics were notable, since the osteogenic and chondrogenic differentiation fates of CESCs may be responsible for the balance between ossification and chondrification in the CEP.

Due to its avascular nature, the microenvironment surrounding the IVD is exposed to hypoxia (14). Hypoxia

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*Abbreviations:* IVD, intervertebral disc; LBP, lower back pain; DDD, degenerative disc disease; CEP, cartilage endplate; MSCs, mesenchymal stem cells; CESCs, cartilage endplate-derived stem cells; DEGs, differentially expressed genes; ASGs, alternatively spliced genes; AS, alternative splicing

*Key words:* cell differentiation, stem cells, hypoxia, gene expression profiles, alternative splicing, high-throughput screening technology

may influence the osteogenesis of mesenchymal stem cells (MSCs) (15), indicating that the physiological hypoxic micro-environment may regulate the osteogenesis of C ESCs, thereby regulating the ossification of the CEP.

During research into the mechanisms through which hypoxia regulates CESC osteogenesis, it was hypothesized that alternative splicing (AS) may serve a 'bridging' role between hypoxia and CESC osteogenesis. The hypothesis may be attributed to numerous observations. Hypoxia may initiate various alternative splicing (AS) events. For example, the inhibitory Per/Arnt/Sim domain protein undergoes unique AS under hypoxic conditions to produce variants in exons 3 and 6, which may establish a novel negative feedback modulation of the adaptive responses to hypoxia/ischemia (16). Furthermore, hypoxia stimulates the generation of the neurotrophin tyrosine kinase receptor type 1 (TrkA) AS variant TrkAIII, which tends to form a stress-resistant phenotype that protects against hypoxia (17). The regulatory roles of AS in stem cell osteogenic differentiation have also been reported. For example, the expression of a TATA binding protein-associated factor 4 (TAF4) variant that does not include exons 6 and 7 in the hTAF4-TAFH domain may promote the early osteogenic differentiation of MSCs (18). In addition, the parathyroid hormone-related protein phenotype becomes selectively increased during the osteogenic differentiation of MSCs, indicating its potential to be a molecular marker of stem cell fate (19).

High-throughput screening technology is a powerful tool that may be used to study the role of gene expression and AS on a genome-wide scale. For example, an exon microarray was previously used to investigate the role of hypoxia on the gene expression profiles and AS events in human umbilical vein endothelial cells (20). In addition, microarray technology was used to identify a hypoxia-associated alternatively spliced laminin-A3 variant that was correlated with poor prognosis, which indicated a decreased probability of survival of 59 patients with head and neck cancer (21).

The present study aimed to investigate the transcriptional and AS mechanisms during CESC osteogenic differentiation under normoxic and hypoxic conditions. The C ESCs were isolated and induced to undergo osteogenic differentiation under normoxic and hypoxic conditions. The samples were extracted and analyzed using the Human Transcriptome Array 2.0 (HTA 2.0) system. An analysis of the gene expression profiles and AS events between the two groups was performed on a genome-wide scale. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) web-based tools were used to analyze the significantly enriched biological processes, molecular functions, cellular components and related signaling pathways of the differentially expressed genes (DEGs) and alternatively spliced genes (ASGs). To the best of our knowledge, genome-wide studies regarding the regulatory role of hypoxia on the transcription and AS events associated with stem cell osteogenic differentiation have not previously been performed; therefore, the present study may be helpful to elucidate the mechanisms underlying the hypoxia-regulated osteogenic differentiation of C ESCs, which is beneficial for increasing understanding of the mechanisms underlying CEP ossification.

## Materials and methods

**Ethics statement.** The CEP samples used in the present study were obtained from patients who experienced disc herniation accompanied by spondylolisthesis and underwent discectomy procedures at the Xinqiao Hospital, Third Military Medical University (Chongqing, China) between January 2014 and June 2014 (Table I). The study procedures were approved by the Ethics Committee of Xinqiao Hospital, Third Military Medical University and were performed in accordance with the Declaration of Helsinki; written informed consent was obtained from each patient.

**Tissue procurement.** The adherent tissue (nucleus pulposus and annulus fibrosus) was carefully removed from the surgically obtained CEPs under a sterilized microscope until a thin layer of cartilage remained, which was washed with sterile 0.1 M PBS. Subsequent to being mechanically homogenized, small portions of the CEP tissue were randomly selected for hematoxylin and eosin staining to eliminate the possibility of pollution from any other residual impurity.

**Cell isolation.** The CEP tissues were mechanically cut into pieces and digested in Dulbecco's modified Eagle's medium (DMEM)/F12 (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) containing 0.2% collagenase II (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 1% fetal calf serum (FCS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) overnight at 37°C. The suspension was filtered through a 70- $\mu$ m cell filter and centrifuged for 5 min at 110 x g at room temperature. Following aspiration of the supernatant, the pellet was resuspended in DMEM/F12 containing 10% FCS and 1% penicillin-streptomycin. The cells were transferred to a 25-cm<sup>2</sup> cell culture flask and cultured at 37°C and 5% CO<sub>2</sub>.

**Agarose culture.** Following the first passage, the cells were recultured in an agarose selection solution, which was established as previously described (13). The culture dishes (Costar; Corning Incorporated, Corning, NY, USA) were precoated with a 1% low melting point agarose solution. A mixture containing 0.5 ml DMEM/F12, 0.5 ml 2% low melting point agarose solution and 1 ml culture medium, containing  $\sim 5 \times 10^4$  suspended CEP cells, was added to the culture dishes. The culture dishes were transferred to a 37°C humidified incubator containing 5% CO<sub>2</sub>. The culture medium was changed twice a week. Following 6 weeks of culturing, the cell aggregates (diameter, >50  $\mu$ m) were transferred to a 25-cm<sup>2</sup> cell culture flask using a sterile Pasteur pipette and were cultured in a 37°C humidified incubator containing 5% CO<sub>2</sub>. The agarose was gradually absorbed into the culture medium and the cells grew as adherent cultures. Cells within passage 3 were used in the present study.

**Induction and oxygen deprivation.** For osteogenic differentiation, the cells were induced in osteogenic induction medium (OIM; HUXMA-90021; Cyagen Biosciences, Inc., Guangzhou, China) under hypoxic conditions (1% O<sub>2</sub>) and normoxic conditions (21% O<sub>2</sub>). The medium was changed twice a week over a period of 3 weeks.

Table I. Patient information.

Case no.	Gender	Age, years	Diagnosis	Degenerated disc level	Surgery type
1	Male	50	Spondylolisthesis	L4-L5	TLIF
2	Male	55	Spondylolisthesis	L4-L5	TLIF
3	Female	52	Spondylolisthesis	L4-L5	TLIF

L, lumbar; TLIF, transforaminal lumbar interbody fusion.

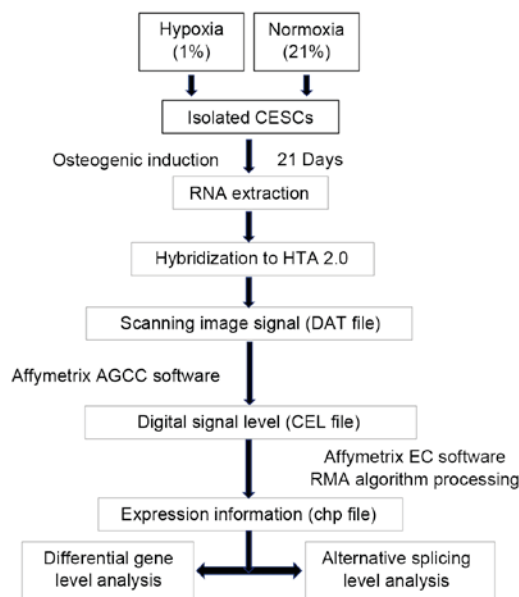


Figure 1. Diagram of the workflow of the present study. The total RNA was extracted from cells subjected to different treatments and hybridized to the Affymetrix HTA 2.0. The AGCC and EC software packages were used to perform the data analysis. HTA, human transcriptome array; AGCC, Affymetrix GeneChip Command Console; EC, expression console; RMA, Robust Multichip Analysis.

**Western blotting.** In order to evaluate the osteogenesis of C ESCs, the protein expression levels of runt-related transcription factor 2 (RUNX2) and collagen type I (COL1) were examined. RUNX2 is the master regulator of osteoblast differentiation and maturation, which is necessary for skeletogenesis (22). COL1, which is the major organic component of bone, may affect the expression of bone cell phenotypes (23,24). RUNX2 and COL1 have been recognized as markers of osteogenic differentiation (25). Cell lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) was used for the extraction of total protein. The protein concentration was determined using a BSA kit (Beyotime Institute of Biotechnology) and 30  $\mu$ g protein from each sample was loaded per lane. The proteins in whole cell lysates were separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were incubated with rabbit anti-human monoclonal antibody against RUNX2 (12556; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA) and mouse anti-human monoclonal antibody against COL1 (ab90395; 1:1,000; Abcam, Cambridge, UK) overnight at 4°C. Following washing three times with PBS, the membrane was incubated with horseradish peroxidase-conjugated horse

anti-mouse (7076; 1:5,000) or goat anti-rabbit polyclonal immunoglobulin G (7074; 1:5,000) (both from Cell Signaling Technology, Inc.) secondary antibodies for 2 h at room temperature. Protein expression was measured via chemiluminescent detection using ECL western blotting reagents (Thermo Fisher Scientific, Inc.). The protein expression levels were normalized to the rabbit antibody against  $\beta$ -actin (4967; 1:5,000; Cell Signaling Technology, Inc.). The protein expression data were analyzed using Quantity One software version 4.6.2 (Bio-Rad Laboratories, Inc.).

**Alizarin red and alkaline phosphatase (ALP) staining.** In order to identify mineral deposits following the different treatments, the cells were fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature, washed 3 times with PBS, stained with alizarin red (Cyagen Biosciences, Inc.) for 5 min at room temperature and washed a further 3 times with PBS. For ALP staining, the cells were fixed with 4% PFA for 30 min at room temperature, stained with an ALP assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol, and washed 3 times with PBS. Subsequently, images of the stained cells were captured.

**Affymetrix HTA 2.0.** Total RNA was extracted from the cell samples using TRIzol extraction (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and hybridized to HTA 2.0 (Affymetrix, Inc., Santa Clara, CA, USA). With probes targeting exons and junctions, HTA 2.0 is able to simultaneously analyze gene expression profiles and AS events. The microarray was scanned by CapitalBio Corporation (Beijing, China), according to the manufacturer's protocol. The image signals of the microarray were saved as DAT files. The Affymetrix GeneChip Command Console software version 3.2 (Affymetrix, Inc.) converted the DAT files (image signals) into CEL files (digital signals). The CEL files were transformed to chp files for quantile normalization, probe set signal integration and background correction with a Robust Multichip Analysis algorithm using the Affymetrix Expression Console software version 1.3 (Affymetrix, Inc.). The DEGs and ASGs in the chp files were subsequently analyzed using the Affymetrix Transcriptome Analysis Console software version 3.0 (Affymetrix, Inc.). Web-based tools including the Database for Annotation, Visualization and Integrated Discovery (david.ncifcrf.gov), KEGG (www.genome.jp/kegg), and Molecule Annotation System (mas.capitalbiotech.online/mas3) were used to identify the significantly enriched GO terms and signaling pathways. The workflow of the present study is presented in Fig. 1.

Table II. Primer sequences.

A, RT-qPCR primers for DEG validation	
Gene symbol	Primer sequence (5'-3')
MMP3-F	AGAAGTGAGCAACTGCAAAAACCT
MMP3-R	CTTCCCCGTCACCTCCAATC
AKR1C1-F	CAATTGAAGCTGGCTTCCCGC
AKR1C1-R	GACCAACTCTGGTCGATGGG
MFGE8-F	AACAGCATCCCTGACAAGCA
MFGE8-R	GGAAGATCTGCAGCCACTGA
CCL2-F	AGCAGCAAGTGTCCTCAAAGA
CCL2-R	GTGTCTGGGGAAAGCTAGGG
FGF7-F	CAGCGTCACAGCAACTGAAC
FGF7-R	TAGTGTAGTGCTCCGGGTGT
POSTN-F	TCCCCGTGACTGTCTATAAGC
POSTN-R	GTGACCTTGGTGACCTCTTCT
GPM6B-F	GCGAGACCTGCAAACTTGTG
GPM6B-R	GTTGCTCAAGAATCGCCACG
IGFBP2-F	CGAGGGCACTTGTGAGAAGC
IGFBP2-R	CAGTGACCTTCTCCCGGAAC
VCAM1-F	GGACCACATCTACGCTGACA
VCAM1-R	TTGACTGTGATCGGCTTCCC
FBLN5-F	TTGTGAGGAGTCTAGCCAGTTG
FBLN5-R	TGGTTTTGCTTAGCCCTCTTCA
β-actin-F	CAACCGGGAAGGAAATGAATGG
β-actin-R	GCCCAATACGACCAAATCAGAG

B, Semi-quantitative PCR primers for ASG validation

Gene symbol	Primer sequence (5'-3')
IFNGR1-F	CTTTCTCCTACCCCTTGT
IFNGR1-R	CCTGTGGCATGATCTGGT
PAX2-F	CCTCCCCCTCCTGTTTCCA
PAX2-R	TGCTGGGTGAAGGTGTCA
BCAP29-F	TTCTAAGGCACAAAATGA
BCAP29-R	GAGGCTAACATAACAAAATT
POSTN-F	AATCCCCGTGACTGTCTA
POSTN-R	ATTGCTTCTTTGTGCTGA
FXR1-F	ACGAAGGACTGATGAAGA
FXR1-R	CTGGAGTACGCTGTAGCT
CACNB4-F	GTTTTACAGCGGTTGATT
CACNB4-R	TGGGGTTTGTAAAGTGCC
TUBD1-F	ACTTGTACCGATCTTCCAG
TUBD1-R	CCAAGTTAGCAATGGAAGTGTTAAA
GPM6B-F	GCGAGACCTGCAAACTTGTG
GPM6B-R	GTTGCTCAAGAATCGCCACG
MEF2C-F	ATCTCCGAGTTCTTATTCC
MEF2C-R	TATCCTCCCATTCTTGT
CADM1-F	GAAATGCCTCAACACGCCGTAC

Table II. Continued.

B, Semi-quantitative PCR primers for ASG validation	
CADM1-R	ACGACGCCACCGATCACG
β-actin-F	CAACCGGGAAGGAAATGAATGG
β-actin-R	GCCCAATACGACCAAATCAGAG

DEG, differentially expressed gene; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; F, forward; R, reverse; ASG, alternatively spliced gene; MMP3, matrix metalloproteinase 3; AKR1C1, aldo-keto reductase family 1 member C1; MFGE8; milk fat globule-EGF factor 8 protein; CCL2, C-C motif chemokine ligand 2; FGF7, fibroblast growth factor 7; POSTN, periostin; GPM6B, glycoprotein M6B; IGFBP2, insulin like growth factor binding protein 2; VCAM1, vascular cell adhesion molecule 1; FBLN5, fibulin 5; IFNGR1; interferon  $\gamma$  receptor 1; PAX2, paired box 2; BCAP29; B-cell receptor associated protein 29; FXR1, FMR1 autosomal homolog 1; CACNB4, calcium voltage-gated channel auxiliary subunit  $\beta$  4; TUBD1, tubulin  $\delta$  1; MEF2C, myocyte enhancer factor 2C; CADM1, cell adhesion molecule 1.

*Criteria for detecting DEGs and ASGs.* The data from the different samples under normoxic conditions were used as the control level for calculating the fold changes in gene expression. Fold changes in gene expression of  $\leq 2$  or  $\geq 2$  and a p-value of  $< 0.05$  were considered to be the threshold for significant DEGs. A splicing index (SI) model was used to determine the ASGs. SI, which represents the ratio of the signal intensity of an exon normalized to that of the target gene between the two experimental groups, was employed to analyze the level of exon exclusion/inclusion. The SI value was obtained using the following formulae:

$$NI(i, j)_A = \frac{\text{exon}_i \text{ signal intensity in condition A}}{\text{gene}_j \text{ signal intensity in condition A}}$$

$$SI(X, Y) = \text{Log}_2 \frac{NI(X, Y)_H}{NI(X, Y)_N}$$

Where NI, normalized intensity;  $NI(i, j)_A$ , the signal intensity of the *i*th exon normalized to that of the *j*th gene in the condition A; N, normoxic induction condition; and H, hypoxic induction condition. The threshold for ASGs was set as SI (linear) values of  $\geq 2/\leq 2$  and a p-value of  $< 0.05$ .

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).* Total RNA was obtained from the cell samples using TRIzol, according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 1  $\mu\text{g}$  RNA from each sample was transcribed into cDNA according to the manufacturer's protocol using the PrimeScript™ RT Master Mix kit (RR047A; Takara Bio, Inc., Otsu, Japan). A total of 1  $\mu\text{g}$  cDNA from each sample was used for qPCR with SYBR Premix Ex Taq™ II (RR820A; Takara Bio, Inc.). The qPCR was a two step-method (without an extension step) according to the manufacturer's protocols, and performed under the following conditions: 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec and 60°C for 34 sec. Following each

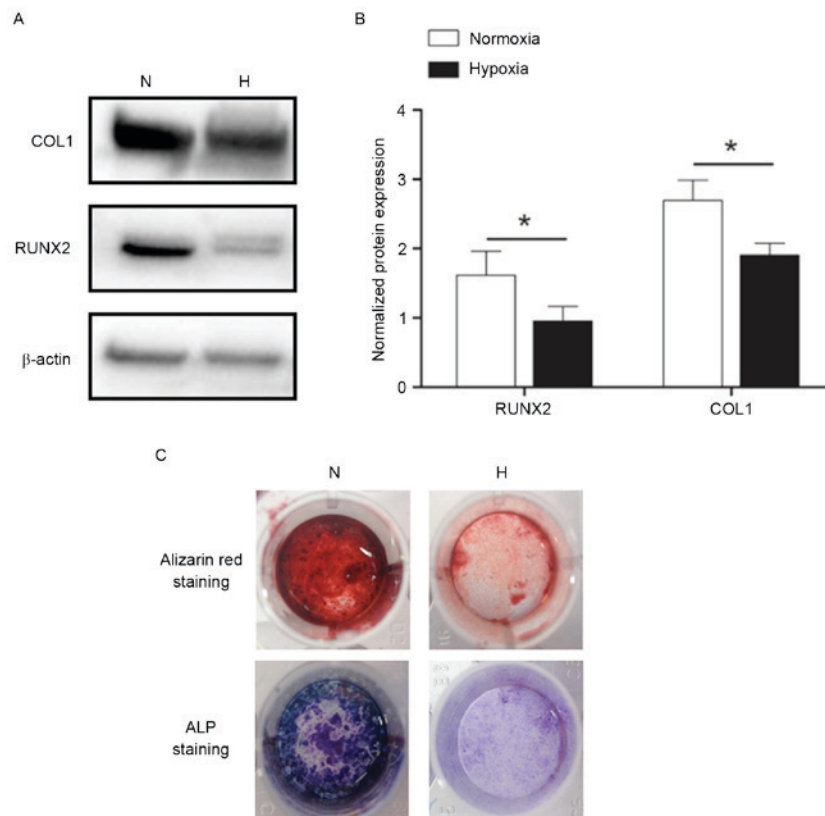


Figure 2. Hypoxia decreases osteogenic differentiation. C ESCs were induced to differentiate into the osteogenic lineage under normoxic and hypoxic conditions for 21 days. (A) Western blotting and (B) densitometric analysis of the expression of RUNX2 and COL1 in the samples. The protein expression levels were normalized to  $\beta$ -actin. Data are presented as the mean  $\pm$  standard deviation;  $n=3/\text{group}$ . \* $P<0.05$ . (C) Macrographs of alizarin red and ALP staining in C ESCs that were induced under normoxic and hypoxic conditions. C ESCs, cartilage endplate-derived stem cells; N, normoxia; H, hypoxia; RUNX2, runt-related transcription factor 2; COL1, collagen type 1; ALP; alkaline phosphatase.

run, dissociation curves were generated using temperatures ranging between 60 and 95°C. The expression levels of each gene were normalized to the levels of  $\beta$ -actin and analyzed using the  $2^{-\Delta\Delta C_q}$  method (26). The sequences of the specific primers are presented in Table II.

**ASG validation by semi-quantitative RT-PCR.** Total RNA extraction and cDNA synthesis were performed as in the aforementioned RT-qPCR method. A total of 2  $\mu\text{l}$  cDNA from each sample was used to conduct semi-quantitative RT-PCR with Premix Taq<sup>TM</sup> (RR901A; Takara Bio, Inc.) and specific primers that were designed to flank the constitutively expressed exons. The sequences of the specific primers are presented in Table II. The expression levels of each ASG were normalized to the expression levels of  $\beta$ -actin. The ASGs of interest were selected for validation according to the following criteria: i) A whole exon skip/gain; ii) an increased absolute SI value; and iii) the first and last AS exons tended to be excluded due to difficulties in designing primers. The semi-quantitative RT-PCR was performed using 1.5% agarose gel and Gold Nucleic Acid Gel Stain (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) under the following conditions: 94°C for 30 sec, followed by 30 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec.

**Statistical analysis.** Data are expressed as the mean  $\pm$  standard deviation for each independent experiment. Comparisons

were made using an independent sample t-test to determine the significance between the two groups.  $P<0.05$  was considered to indicate a statistically significant difference. All data were analyzed with SPSS version 19.0 (IBM Corp., Armonk, NY, USA).

## Results

**Hypoxia inhibits the osteogenic differentiation of C ESCs.** In order to evaluate the effects of hypoxia on osteogenic differentiation, C ESCs were stimulated to differentiate into the osteogenic lineage under normoxic (21%  $\text{O}_2$ ) and hypoxic (1%  $\text{O}_2$ ) conditions for 21 days. The protein expression levels of osteogenic differentiation markers RUNX2 and COL1 in the hypoxia group were decreased compared with in the normoxia group (Fig. 2A and B). In addition, hypoxia exhibited an inhibitory effect on the functional mineralization of C ESCs (Fig. 2C). These findings suggested that the osteogenesis of C ESCs was inhibited in the hypoxic microenvironment.

**DEG detection, validation and functional analysis during osteogenic differentiation of C ESCs under normoxic and hypoxic conditions.** A comparative genome-wide analysis of the DEGs in the hypoxia and normoxia groups identified 214 DEGs, of which 95 (44%) were upregulated and 119 (56%) were downregulated. In addition, 56 (26%) were non-coding transcripts. A total of 10 DEGs were selected for validation

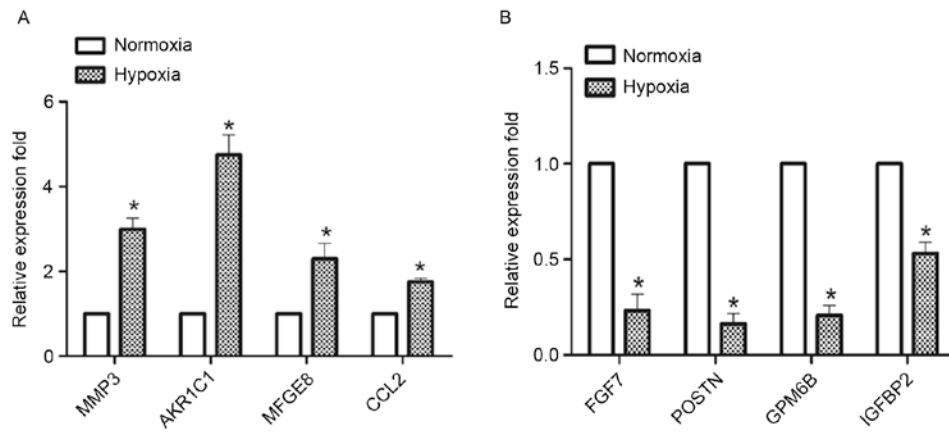


Figure 3. DEGs in CESC*s* during osteogenic differentiation under normoxic and hypoxic conditions were validated using RT-qPCR. CESC*s* were induced to differentiate in osteogenic induction medium under normoxic and hypoxic conditions for 21 days. A total of 8 of the 10 DEGs were validated by RT-qPCR. (A) MMP3, AKR1C1, MFGE8, CCL2, and (B) FGF7, POSTN, GPM6B and IGFBP2. The expression levels of the genes were normalized to  $\beta$ -actin, and the data are presented as the mean  $\pm$  standard deviation; n=3/group. \*P<0.05 vs. respective normoxia group. DEGs, differentially expressed genes; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CESC*s*, cartilage endplate-derived stem cells; MMP3, matrix metalloproteinase 3; AKR1C1, aldo-keto reductase family 1 member C1; MFGE8, milk fat globule-EGF factor 8 protein; CCL2, C-C motif chemokine ligand 2; FGF7, fibroblast growth factor 7; POSTN, periostin; GPM6B, glycoprotein M6B; IGFBP2, insulin like growth factor binding protein 2.

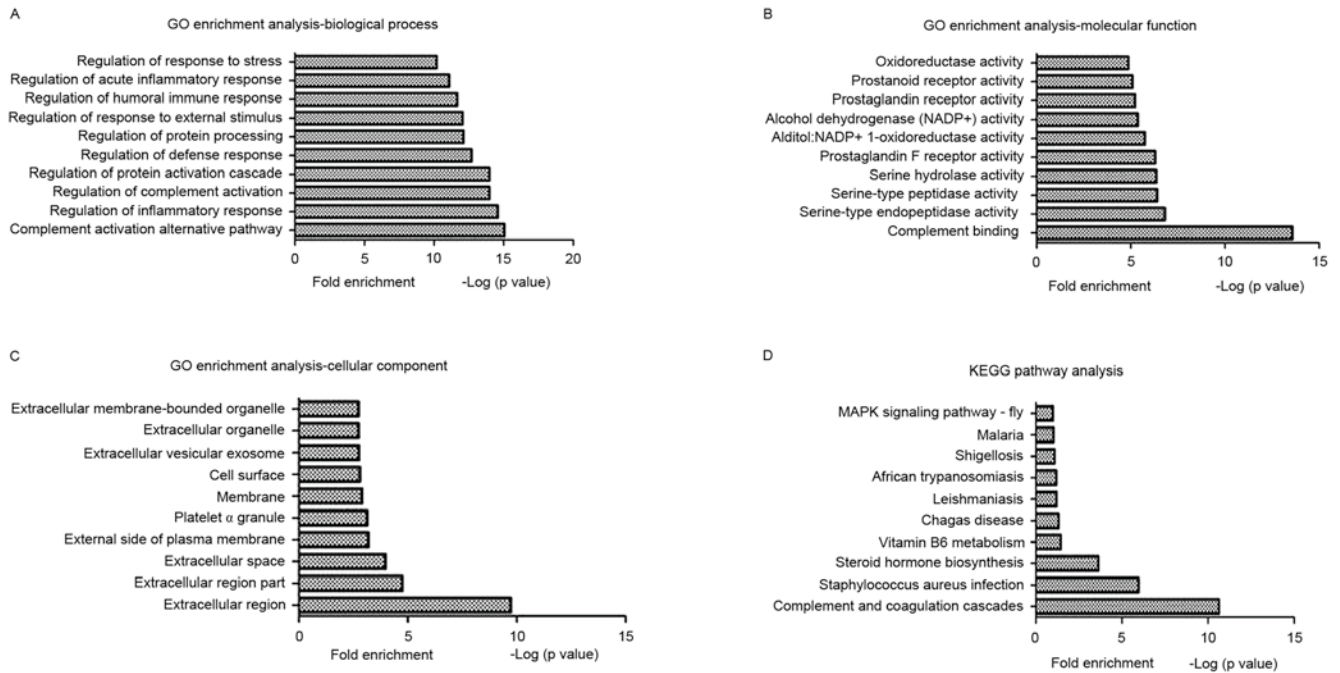


Figure 4. GO function and KEGG pathway enrichment in the differentially expressed genes during osteogenic differentiation of CESC*s* under normoxic and hypoxic conditions. The top ten GO functions that were enriched in the (A) biological process, (B) molecular function and (C) cellular component categories, and (D) the top ten KEGG pathways that were enriched during the osteogenic differentiation of CESC*s* under normoxic and hypoxic conditions. GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; CESC*s*, cartilage endplate-derived stem cells.

and 8 of them were validated (consistent tendency). The expression levels of matrix metalloproteinase 3, aldo-keto reductase family 1 member C2, milk fat globule EGF-factor 8 protein and C-C motif chemokine ligand 2 were upregulated, whereas the expression levels of fibroblast growth factor 7 (FGF7), periostin (POSTN), glycoprotein M6B (GPM6B) and insulin like growth factor binding protein 2 (IGFBP2) were downregulated in the hypoxia group compared with the normoxia group (Fig. 3). Two of the genes [vascular cell adhesion molecule 1 (VCAM1) and fibulin 5 (FBLN5)] were not differentially expressed (data not presented).

A GO enrichment analysis of the DEGs during osteogenic differentiation of CESC*s* under normoxic and hypoxic conditions was performed to identify the enriched biological processes, molecular functions and cellular components. The results of the present study demonstrated that certain important GO terms were significantly enriched, including complement activation (alternative pathway), complement binding and the extracellular region. The top 10 GO functions that were enriched in the DEGs during the osteogenic differentiation of CESC*s* under normoxic and hypoxic conditions are presented in Fig. 4A-C.

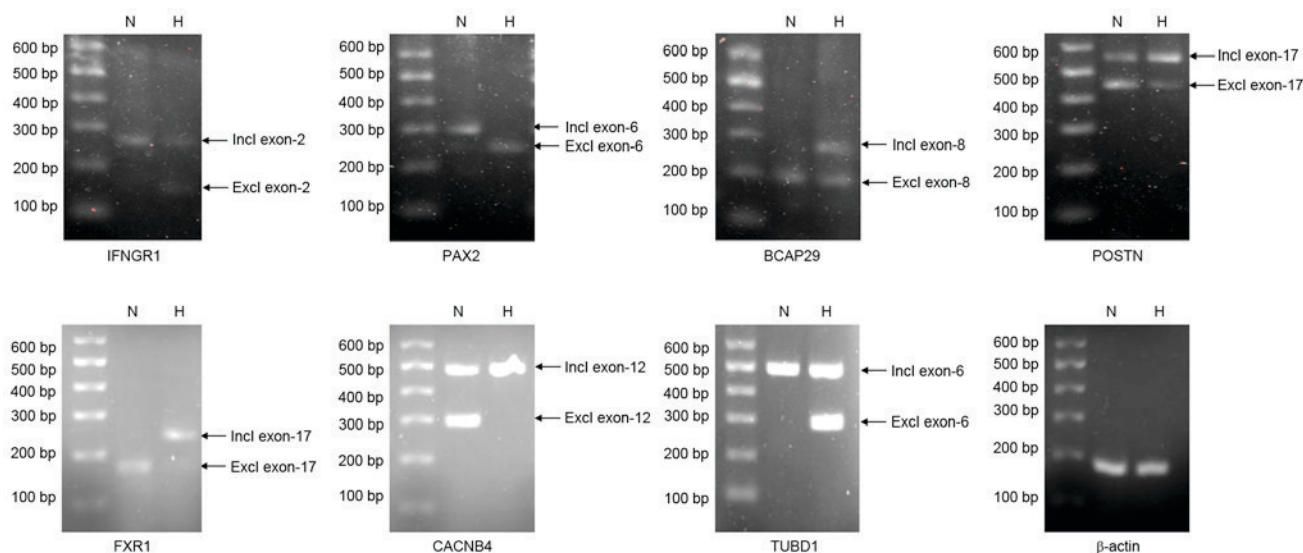


Figure 5. Alternatively spliced genes during the osteogenic differentiation of CESC under normoxic and hypoxic conditions were validated by semi-quantitative PCR. CESC were induced to differentiate in osteogenic induction medium under normoxic and hypoxic conditions for 3 weeks. A total of 7 of the 10 ASGs were successfully validated by semi-quantitative PCR.  $\beta$ -actin was used as an internal control. CESC, cartilage endplate-derived stem cells; PCR, polymerase chain reaction; N, normoxia; H, hypoxia; IFNGR1, interferon  $\gamma$  receptor 1; PAX2, paired box 2; BCAP29, B-cell receptor associated protein 29; POSTN, periostin; FXR1, FMR1 autosomal homolog 1; CACNB4, calcium voltage-gated channel auxiliary subunit  $\beta$  4; TUBD1, tubulin  $\delta$  1; Incl, inclusion; Excl, exclusion.

The KEGG web-based tool was used to identify the functional pathways that were significantly enriched in the DEGs. The results revealed that numerous cellular pathways were involved, including the complement and coagulation cascades, *Staphylococcus aureus* infection and steroid hormone biosynthesis. The top 10 KEGG pathways that were enriched in the DEGs during the osteogenic differentiation of CESC under normoxic and hypoxic conditions are presented in Fig. 4D.

**ASG detection, validation and functional analysis during the osteogenic differentiation of CESC under normoxic and hypoxic conditions.** The analysis of genome-wide AS events identified 6,999 AS exons belonging to 1,618 ASGs during the osteogenic differentiation of CESC under normoxic and hypoxic conditions. A total of 3,227 (46%) AS exons with SI values  $\geq 2$  were defined as 'general exon inclusion' events, whereas the remaining 3,772 (54%) AS exons were defined as 'general exon exclusion' events. Each ASG exhibited 4.33 (6,999/1,618) AS exons on average, confirming that numerous AS events may occur in the same gene. POSTN was selected as a typical example, which exhibited 21 AS exons, indicating a complex regulatory effect of AS. Notably, 42 of these 1,618 ASGs were also significant DEGs, which indicated an underlying intrinsic relationship between AS and gene expression. In order to validate their accuracy, 10 ASGs were selected for the semi-quantitative RT-PCR analysis. The data presented in Fig. 5 demonstrated that 7 of the 10 selected ASGs were successfully validated.

A GO enrichment analysis of the ASGs during the osteogenic differentiation of CESC under normoxic and hypoxic conditions was performed to identify the enriched biological processes, molecular functions and cellular components. The results of the present study demonstrated that certain GO terms were regulated by AS in CESC under hypoxic conditions, including DNA-dependent regulation of transcription,

protein binding and cytoplasm. The top 10 GO terms of the ASGs during the osteogenic differentiation of CESC under normoxic and hypoxic conditions are presented in Table III.

The 1,618 ASGs were analyzed with the KEGG web-based tool to identify the enriched signaling pathways. The results demonstrated that numerous signaling pathways were significantly affected, including focal adhesion, ubiquitin-mediated proteolysis and the mitogen-activated protein kinase signaling pathway. The top 10 KEGG pathways that were enriched in the ASGs during the osteogenic differentiation of CESC under normoxic and hypoxic conditions are presented in Table IV.

## Discussion

CESC are considered to be MSCs due to their embryonic mesoderm origin, similar surface immunophenotypes and similar differentiation capacity compared with other reported MSCs (12).

The *in situ* environment of MSCs is frequently hypoxic: Bone marrow, 4-7%  $O_2$ ; muscle, 1-10%  $O_2$ ; and adipose tissue, 3.8-9.6%  $O_2$  (27,28). In response to hypoxia, MSCs exhibit altered differentiation fates according to their different tissues of origin. For example, in bone marrow MSCs, the hypoxic microenvironment has been demonstrated to favor chondrogenesis, and inhibit osteogenesis and adipogenesis (29-31). In adipose MSCs, adipogenesis and chondrogenesis have been observed to be promoted, whereas osteogenesis was reduced under hypoxic conditions (15,32). In muscle MSCs, the decreased oxygen level improved myogenic differentiation and inhibited adipogenic differentiation (22). In addition, periodontal ligament MSCs exhibited increased osteogenic differentiation under hypoxic conditions (33). Therefore, stem cells derived from various tissues exhibit a tissue-specific differentiation fate in response to physiological hypoxia, which may be beneficial for their physiological functions.

Table III. List of top ten enriched GO terms according to GO analysis of alternatively spliced genes.

GO term	Count	P-value
<b>Biological process</b>		
GO:0006355 regulation of transcription, DNA-dependent	133	3.69x10 <sup>-104</sup>
GO:0006350 transcription	114	1.30x10 <sup>-77</sup>
GO:0007049 cell cycle	64	1.15x10 <sup>-57</sup>
GO:0006468 protein amino acid phosphorylation	57	2.01x10 <sup>-54</sup>
GO:0007165 signal transduction	101	1.93x10 <sup>-50</sup>
GO:0055114 oxidation reduction	48	5.60x10 <sup>-45</sup>
GO:0051301 cell division	37	6.57x10 <sup>-45</sup>
GO:0019941 modification-dependent protein catabolism	46	4.68x10 <sup>-44</sup>
GO:0007067 mitosis	29	7.80x10 <sup>-34</sup>
GO:0007155 cell adhesion	42	5.84x10 <sup>-33</sup>
<b>Molecular function</b>		
GO:0005515 protein binding	504	0
GO:0008270 zinc ion binding	206	1.30x10 <sup>-202</sup>
GO:0000166 nucleotide binding	184	4.57x10 <sup>-182</sup>
GO:0046872 metal ion binding	222	1.73x10 <sup>-171</sup>
GO:0005524 ATP binding	154	6.58x10 <sup>-169</sup>
GO:0016740 transferase activity	130	7.67x10 <sup>-120</sup>
GO:0005509 calcium ion binding	82	1.78x10 <sup>-80</sup>
GO:0003677 DNA binding	99	4.12x10 <sup>-66</sup>
GO:0003723 RNA binding	62	6.92x10 <sup>-61</sup>
GO:0004674 protein serine/threonine kinase activity	47	2.39x10 <sup>-51</sup>
<b>Cellular component</b>		
GO:0005737 cytoplasm	453	0
GO:0005634 nucleus	455	0
GO:0016020 membrane	265	1.01x10 <sup>-163</sup>
GO:0016021 integral to membrane	212	6.83x10 <sup>-136</sup>
GO:0005829 cytosol	108	1.08x10 <sup>-117</sup>
GO:0005886 plasma membrane	150	3.25x10 <sup>-98</sup>
GO:0005576 extracellular region	116	2.69x10 <sup>-90</sup>
GO:0005794 Golgi apparatus	69	1.30x10 <sup>-65</sup>
GO:0005783 endoplasmic reticulum	69	2.45x10 <sup>-63</sup>
GO:0005739 mitochondrion	71	1.01x10 <sup>-61</sup>

GO, Gene Ontology.

In the present study, OIM was used under both hypoxic and normoxic conditions. Under normoxic conditions, Huang *et al.* (34) reported that OIM was able to significantly induce the osteogenic differentiation of C ESCs, compared with normal growth medium. It was observed in Huang *et al.* (34) study that the expression of osteogenic differentiation marker genes (RUNX2, ALP and OC) was decreased in the absence of OIM under normoxic conditions. In addition, previous studies have demonstrated that stem cell differentiation may be decreased in hypoxic conditions compared with normoxic

Table IV. List of top 10 signaling pathways enriched by Kyoto Encyclopedia of Genes and Genomes analysis of alternatively spliced genes.

Pathway	Count	P-value
Focal adhesion	31	4.68x10 <sup>-18</sup>
Ubiquitin mediated proteolysis	25	1.16x10 <sup>-16</sup>
MAPK signaling pathway	29	7.75x10 <sup>-13</sup>
ECM-receptor interaction	15	1.64x10 <sup>-10</sup>
Regulation of actin cytoskeleton	23	1.78x10 <sup>-10</sup>
Axon guidance	18	2.00x10 <sup>-10</sup>
Insulin signaling pathway	17	3.97x10 <sup>-9</sup>
Leukocyte transendothelial migration	16	4.01x10 <sup>-9</sup>
Complement and coagulation cascades	12	1.51x10 <sup>-8</sup>
Cell adhesion molecules	16	1.78x10 <sup>-8</sup>

MAPK, mitogen-activated protein kinase; ECM, extracellular matrix.

conditions (35,36). Therefore, in the absence of OIM, osteogenic differentiation occurs at a low level, and this is physiologically important for C ESCs to maintain their stem cell properties. In the research area of hypoxia/normoxia, normal growth medium is frequently used to investigate growth, including proliferation and apoptosis (37,38), whereas OIM is used to study the potential for osteogenesis (15,30). The present study focused on the potential of stem cells to undergo osteogenesis; therefore, OIM was used in the present study.

Anatomically, the CEPs of healthy, mature juvenile, adolescent and adult discs are free of blood vessels, whereas the bone endplate (BEP, part of the vertebral bone) contains extensive blood spaces for the vertebral blood sinuses. Blood vessels end at the interface between the CEP and BEP, without invading the CEP (39). Lee *et al.* (40) reported that all three components of the IVD (nucleus pulposus, annulus fibrosus and CEP) remained in a hypoxic microenvironment in a rat model using the 2-nitroimidazole, EF5, a drug that forms covalent adducts with cellular proteins at low oxygen concentrations. During the process of degeneration, blood vessels may invade into the CEP through fissures (39,41,42). Blood vessel invasion may increase the oxygen levels, which may disrupt the physiological hypoxic microenvironment of the C ESCs. This destruction of the hypoxic microenvironment may facilitate the osteogenic differentiation of C ESCs, which may initiate the ossification of the CEP. The ossification of the CEP has been demonstrated to result in a poor capacity to resist mechanical stress and poor nutrient exchange, and, therefore, to initiate IVD degeneration.

The present study sought to elucidate the mechanism through which hypoxia inhibits the osteogenic differentiation of C ESCs. At present, genome-wide transcription analysis is the predominant method used to study the mechanism of stem cell differentiation. Previous studies have used this tool to obtain a coherent view of the transcriptional and post-transcriptional alterations during the differentiation process (43-46). However, to the best of our knowledge, no previous studies have investigated the AS mechanisms of stem cell differentiation under hypoxic conditions on a genome-wide scale. AS is considered



to be an intricate regulatory mechanism through which a single pre-mRNA may produce various mature RNA subtypes, which leads to structural diversity of the genetic phenotypes without expansion of the genome (47). Since hypoxia is a source of AS events, and AS is associated with the regulation of the osteogenic differentiation of stem cells, it was hypothesized that AS may be the connection between hypoxia and the chondrogenic differentiation of C ESCs (16-18). Therefore, the present study used high-throughput screening technology to identify DEGs and ASGs during the osteogenic differentiation of C ESCs under normoxic and hypoxic conditions, and analyzed the GO enrichment terms and functional pathways with relevant web-based bioinformatics tools.

The detected DEGs between the normoxic osteogenic induction and hypoxic osteogenic induction groups were validated by qPCR. FGF7, GPM6B and IGFBP2, which were downregulated in the hypoxia group compared with the normoxia group, were used as examples. FGF7 may facilitate the dexamethasone-,  $\beta$ -glycerophosphate- and ascorbic acid-induced osteogenic differentiation of embryonic stem cells into bone-like nodules and induce mineralization through the extracellular signal-related kinase/RUNX2 signaling pathway (48). GPM6B, which encodes a membrane glycoprotein of the proteolipid protein family, is upregulated during osteoblast differentiation. GPM6B silencing in MSCs was observed to lead to decreased mineralization of the extracellular matrix and reduced ALP activity; a microarray analysis attributed these alterations to cytoskeleton and matrix vesicle release (49). IGFBP2 was reported to trigger the osteogenic differentiation of MSCs by interacting with integrin  $\alpha$  5 and insulin like growth factor 2 (50). These results suggested that FGF7, GPM6B and IGFBP2, which were downregulated in the present study, may be associated with the osteogenesis of C ESCs, confirming the inhibition of osteogenic differentiation by hypoxia.

KEGG and GO analysis of the identified DEGs was performed in the present study. The results demonstrated that the complement and coagulation cascades were enriched in the DEGs, according to the KEGG analysis. In addition, in the GO analysis of the DEGs, the biological process of complement activation (alternative pathway) and the molecular function of complement binding were enriched. The results of the present study indicated that the complement pathway may be associated with the mechanism through which hypoxia is able to regulate the osteogenic differentiation of C ESCs.

Due to the avascular nature and low immunogenicity of the IVD, few studies have investigated the role of immune signaling in the CEP. However, blood vessel invasion may be observed in the degenerated CEP, and revascularization may provide immune signals (30,31). Furthermore, numerous immune signals have been observed to be involved in a series of non-immune effects. In the present study, the complement pathway was used as an example. The complement pathway is known to exhibit roles in immune surveillance, and has previously been demonstrated to be involved in non-immune effects, including angiogenesis, clearance of apoptotic cells, and stem cell recruitment and differentiation (51). MSCs and osteoblasts express proteins in the complement cascade (52), including complement C3, C3a anaphylatoxin chemotactic receptor (C3aR), complement C5, C5a anaphylatoxin chemotactic receptor (C5aR), and cell

surface markers, including cluster of differentiation (CD)46, CD55 and CD59 (53). C5aR expression was markedly increased during osteogenic differentiation in MSCs (54), and osteogenesis was reported to be accelerated in the presence of C5a and C3a in a C5aR- and C3aR-specific manner (41). In the present study, the expression of C3, the precursor of C3a, in the hypoxic group was reduced compared to the normoxic group, indicating a possible method by which hypoxia inhibits osteogenesis of C ESCs. As previously mentioned, it can be hypothesized that the complement cascade was enriched according to GO and KEGG analysis. The precise and complicated regulation of complement cascade on osteogenesis may include both enhancement and suppression. Since hypoxia is an important activator of the complement pathway (55,56), complement activation may serve a role in regulating the osteogenic differentiation of C ESCs under hypoxic conditions.

A total of 7 out of the 10 chosen ASGs were successfully validated. In the present study, interferon  $\gamma$  (IFN $\gamma$ ) receptor 1 (IFNGR1) was used as an example to elucidate the role of AS. The exclusion of exon 2 of IFNGR1 was reported to be a characteristic of IFNGR1 deficiency disease, in which patients exhibited impaired IFN $\gamma$ -mediated function, leading to susceptibility to infection (57). In the present study of the osteogenic differentiation of C ESCs, the exclusion of exon 2 of IFNGR1 was detected in the hypoxia group compared with in the normoxia group. IFNGR1 is the receptor for IFN $\gamma$ ; the IFN $\gamma$ /IFNGR1 complex is responsible for activation of the IFN pathway. IFN $\gamma$  was reported to promote osteogenic differentiation *in vitro* (58) and *in vivo* (59), whereas the osteogenesis of allogeneic MSCs was inhibited by IFN $\gamma$  (60). Given that exon 2 exclusion may lead to the change of IFN $\gamma$ -mediated function, it is reasonable to consider that exon 2 exclusion may also influence the IFN $\gamma$ -mediated regulation of osteogenesis. In conclusion, since exon 2 exclusion occurs in the hypoxia-regulated osteogenesis system and the immune system, it may be hypothesized that immune signals may be associated with immunomodulation, in addition to exhibiting non-immune effects, including osteogenesis regulation, through AS under hypoxic conditions. At present, studies on the role of ASG in hypoxia-regulated osteogenesis remain scarce. However, the results of the present study may offer useful insights for further studies into the role of AS in the mechanism of hypoxia-regulated osteogenesis.

KEGG and GO analyses of the ASGs were performed. Consistent with the high-throughput analysis of the DEGs, the complement and coagulation cascades were enriched in the KEGG analysis. A previous study reported that AS may influence the complement cascade by producing various protein phenotypes (61); therefore, it was hypothesized that AS may be associated with the complement pathway during the process of hypoxia-regulated osteogenesis. In addition, the biological process of DNA-dependent regulation of transcription and the molecular function of nucleotide binding were enriched. A number of the regulatory molecules that are expressed during the osteogenic differentiation process, including RUNX2 and osterix, are transcription factors that function by binding to the promoter of target genes to promote or inhibit transcription (62). In addition, the cellular components cytoplasm and nucleus were enriched, which indicated an increased rate of cytoplasmic/nuclear translocation. A number of molecules

with regulatory effects undergo cytoplasmic/nuclear translocation, including nuclear factor- $\kappa$ B (63) and AKT (64). The GO analysis of the ASGs performed in the present study indicated that AS may be associated with nuclear signal transduction, which may be the functional factor that is influenced by AS during the hypoxia-regulated osteogenesis of CESC.

CEP chondrocytes (CEPCs) are present in the CEP. Therefore, further studies are required to analyze the role of hypoxia in the differentiation/AS regulation of CEPCs. CEPCs are a type of terminally differentiated chondrocyte. To the best of our knowledge, few previous studies have focused on the differentiation properties of terminally differentiated cells. However, hypoxia is reported to induce dedifferentiation to drive committed cells towards a pluripotent fate (65); therefore, hypoxia may serve a role in transforming CEPCs into CESC. A previous study demonstrated the regulatory effects of AS on chondrocytes in response to hypoxia. For example, vascular endothelial growth factor (VEGF)<sub>120</sub> and VEGF<sub>164</sub> are the most abundant splicing variants that are expressed in chondrocytes in response to decreased oxygen levels (66). Therefore, hypoxia/normoxia may regulate AS in CEPCs; however, the specific mechanisms require further study.

In conclusion, the results of the present study demonstrated that hypoxia inhibited osteogenesis in CESC. Alterations in the gene expression profiles and AS events were observed on a genome-wide scale. The subsequent GO and KEGG analyses provided a reference for future mechanistic studies of the gene expression profiles and AS regulation during the inhibition of osteogenesis. Notably, the identification of the significance of the complement pathway in the hypoxia-mediated regulation of osteogenic differentiation will be of use in improving the understanding of this physiological phenomenon, and may be important for the identification of targets for CEP degeneration therapy.

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