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Global profiling of the gene expression and alternative splicing events during hypoxia-regulated chondrogenic differentiation in human cartilage endplate-derived stem cells

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ABSTRACT

The intervertebral disc (IVD) degeneration is initiated by cartilage endplate (CEP) degeneration and is characterised by reduced chondrification. Cartilage endplate-derived stem cells (CESCs) with chondrogenic differentiation abilities are responsible for the restoration of cartilage. CEP remains in an avascular and hypoxic microenvironment. In this study, we observed that the physiological hypoxia greatly promotes the chondrogenic differentiation of CESCs. This tissue specificity of the differentiation fate of CESCs in response to the hypoxic microenvironment was physiologically significant for the CEP to maintain the chondrification status. To investigate the mechanisms underlying the hypoxia-regulated chondrogenic differentiation of CESCs, we adopted a high-throughput scanning technology to detect the global profiling of gene expression and alternative splicing (AS) event changes during chondrogenic differentiation under hypoxia in CESCs compared to those induced under normoxia. An Affymetrix Human Transcriptome Array 2.0 was used to identify the differentially expressed genes (DEGs) and alternatively spliced genes (ASGs). After RT-PCR validation, GO and KEGG pathway analyses of both the DEGs and ASGs were performed. The enrichment of the GO functional terms and signalling pathways provided referential direction of the mechanism to study the gene expression and AS in the hypoxia-regulated chondrogenesis promotion, which could be helpful in understanding this physiological phenomenon, and it could also be instrumental in finding targets for CEP degeneration therapy.

1. Introduction

Degenerative disc disease (DDD) is the most important reason for low back pain (LBP), which usually leads to chronic disabilities [1]. Many pathological factors are responsible for DDD, such as matrix degradation, the invasion of inflammatory factors and cell senescence [2–4], and a decreased metabolic exchange is known as the most important mechanism due to the poor exchange of waste products and nutrition [5]. The intervertebral disc (IVD) is an avascular organ, and its metabolic exchange predominantly depends on the diffusion effect through the cartilage endplate (CEP) in a mature IVD [6]. The CEP is a thin layer of hyaline cartilage that separates the vertebral body from the IVD. Blood vessels are within the adjacent vertebral body end at the interface between the vertebrae and the IVD and do not reach the inner part of the discs [7]. As the CEP is the most important channel of the metabolic exchange, many researchers believe that the degenerative CEP may initiate DDD [8].

Chondrification status is critical in the normal physiological function of the CEP. The cartilaginous biochemical content is not only necessary for the resistance of compressive forces but is also instrumental to the transport characteristics of the CEP. In addition, CEP ossification decreases the solute transport, which finally leads to DDD [9]. However, the mechanisms underlying the regulation of the chondrification status of the CEP still remain unclear.

Recent studies by our team demonstrated the existence of stem cells in the CEP. These cartilage endplate-derived stem cells (CESCs) showed superior ability of chondrogenic differentiation to that of bone marrow mesenchymal-derived stem cells (BM-MSCs) [10]. The differentiation property drew our attention to the possibility that the CESCs may play important roles in cartilage regeneration and restoration in the CEP, and the chondrogenic differentiation status of the CESCs may be responsible for chondrification of the CEP.

As an avascular organ, the CEP remains in a hypoxic microenvironment [11]. Hypoxia greatly impacts the chondrogenic differentiation of MSCs [12], indicating that the physiological hypoxic

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

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microenvironment might regulate the chondrogenesis of the CESCs and thus, regulate the chondrification status of the CEP.

In the process of investigating the relationship between hypoxia and CESC chondrogenic differentiation, we noticed that, in addition to the changes in gene expression, alternative splicing (AS) might also be the middle-link between them by which the regulatory effect of hypoxia is performed. The assumption can be attributed to two reasons: (1) plenty of AS events could be initiated in response to hypoxia; for example, as important negative regulator of the hypoxia-inducible transcription factors, IPAS forms unique AS variants of exons 3 and 6 under hypoxic conditions, which defines a novel action mode of hypoxia-mediated regulation of gene expression [13]. Stimulation of TrkAIII AS by the hypoxic condition, as well as the ability of TrkAIII to generate a stress-resistant and angiogenic phenotype, suggests a protective role against hypoxia [14]. (2) Recently, many studies focus on the role of AS in chondrogenic differentiation of the stem cell. For example, the knockdown of the hTAF4-TAFH domain from the TAF4 phenotype promotes the chondrogenic differentiation of MSCs [15]. Additionally, a novel AT-qPCR method was developed to quantify the phenotypes of the alternatively splicing COl2A1 variant, which is helpful in identifying the chondroprogenitors induced by the chondrogenic induction medium [16]. Given that hypoxia is an important source of AS events and that AS might regulate the chondrogenic differentiation of stem cells, we hypothesise that AS might serve as a regulatory "bridge" between hypoxia and the chondrogenic differentiation of CESCs.

High-throughput screening technology is a prevalent tool to identify the role of gene expression and AS on a genome-wide scale. Hang et al. revealed the transcription and AS regulation in human umbilical vein endothelial cells under hypoxia by an exon microarray [17]. Carla et al. identified a hypoxia-related LAMA3 splice variant that correlated with a poor prognosis by using microarry technology [18]. In our study, we investigated the underlying transcription and AS mechanisms during CESC chondrogenic differentiation under hypoxia. The isolated CESCs were induced to undergo chondrogenic differentiation under normoxia and hypoxia, respectively. Then, the samples were detected and analysed on a genome-wide scale using the Affymetrix Human Transcriptome Array 2.0 (HTA 2.0) system. A comparative analysis of the differentially expressed gene (DEG) profiling and the AS events between the two groups was performed. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were used to construct a functional enrichment of the genes of interest to explain the transcription and AS mechanisms. To our knowledge, a genome-wide study of the regulatory role of hypoxia on the DEG and AS events of chondrogenic differentiation in stem cells has not reported before; thus, our work may illuminate the role of the physiological hypoxic microenvironment in cartilage restoration of the CEP, which is beneficial for understanding the mechanism of DDD and the innovations of new therapies.

2. Materials and methods

2.1. Ethics statement

The CEP samples used in this study were obtained from six patients who underwent discectomy and fusion surgeries in the Department of Orthopaedics at the Xinqiao Hospital (see Supplementary Table 1 in [19]). All of the procedures were in accordance with the Helsinki Declaration, and written informed consent was obtained from each patient.

2.2. CESC isolation and culture

The CEP tissues were minced into small pieces and then digested with 0.2% collagenase II (Sigma, USA) in a DMEM/F12 medium (Hyclone, USA) containing 1% fetal calf serum (FCS) overnight at 37 °C. The suspended cells were then filtered through a 70-μm cell filter to remove the large aggregates. The suspension was centrifuged at 1000 rpm/min for 5 min. The cell pellet was resuspended in a culture medium containing DMEM/F12, 1% penicillin-streptomycin and 10% FCS. Finally, the CEP cells were transferred into a 25-cm^2 cell culture flask and cultured at 37 °C in 5% CO_2. After the first passage of expansion, the cells were transferred to an agarose selection solution. The agarose selection system was used as previously described [10]. Briefly, 60 mm-diameter sized culture dishes (Costar Corning, USA) were coated with 1% low melting point agarose. Then, 0.5 ml of 2% low melting point agarose, 0.5 ml of a DMEM/F12 medium and 1 ml of a culture medium containing 5×10^4 CEP cells was transferred to the culture dishes, and the cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C. The culture medium was changed twice a week. After 6 weeks, the cell aggregates of a diameter larger than 50 μm were isolated with a sterile Pasteur pipette and then transferred into a 25-cm^2 culture flask. Passage 3 cells were used in this study.

2.3. Induction and oxygen deprivation

For chondrogenic differentiation, the cells were induced in chondrogenic induction medium (Cyagen, USA). The induction medium was changed twice a week for a 21-day period. For the hypoxic culture, the CESCs were cultured in 1% O_2. For the normoxic culture, the CESCs were cultured in 21% O_2.

2.4. Western blot

The proteins in the whole cell lysate were separated using SDS-PAGE (Willget Biotech, China) and transferred onto a PVDF membrane (Bio—Rad, USA). The membranes were incubated with the primary antibodies SOX9 and COL2 (Abcam, USA) overnight at 4 °C and then washed three times with PBS. Horseradish peroxidase-conjugated IgG was used as the secondary antibody. Finally, the membranes were subjected to the ECL system (Pierce, USA). The expressions of the proteins were normalised to the Actin-β level.

2.5. Affymetrix Human Transcriptome Array 2.0

The CESCs were induced into chondrogenic differentiation under normoxia and hypoxia, respectively. After 21 days, the cells were treated with Trizol. Total RNA was extracted and hybridised to HTA 2.0 from the Affymetrix Corporation. With probes targeting exons and junctions within the genes, the HTA 2.0 analysed gene expression and AS information simultaneously. The hybridisation and scanning of the microarray were performed according to the recommended protocols by the CapitalBio Corporation (Beijing, China). Briefly, the fluorescence signals of the microarray were obtained as DAT files. The Affymetrix GeneChip® Command Console (AGCC) software converted the DAT image signals into CEL digital signals. Next, the Affymetrix Expression Console software was used to treat the CEL files through a Robust Multichip Analysis (RMA) algorithm, which included probeset signal integration, background correction and quantile normalisation. Then, the CHP files were transferred to the Affymetrix Transcriptome Analysis Console software to analyse the differentially expressed genes (DEGs) and the alternatively spliced genes (ASGs). To identify the significant enrichment of the gene ontology (GO) terms and the functional pathways, DAVID (http://david.abcc.ncifcrf.gov/tools.jsp), Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/), and Molecule Annotation System (MAS) were used. The workflow of the study is shown in Fig. 1. The results of the GO and KEGG analyses are described in the supplementary materials and are presented as tables or figures in this paper.