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Transcriptome changes during TNF-α promoted osteogenic differentiation of dental pulp stem cells (DPSCs)

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ABSTRACT

Dental pulp stem cells (DPSCs), due to the ease of isolation and their capacities of multi-lineage differentiation, are considered as attractive resources for regenerative medicine. In a previous study, we showed that TNF-α promoted the osteogenic differentiation of DPSCs via the NF-κB signaling pathway. However, the mechanisms of such differentiation were largely unknown. Here, we examined the gene expression profiles between undifferentiated, partially differentiated and fully differentiated DPSCs induced by TNF-α by performing the next-generation sequencing technique (RNA-Seq). Our results revealed a continuous transition of the transcriptome changes during TNF-α promoted osteogenic differentiation of DPSC. Bioinformatics analysis revealed a relatively general to specific transformation of the involved signaling pathways from the early to late stages of differentiation. Gene regulatory network analysis highlighted novel, key genes that are essential for osteogenic differentiation at different time points. These results were further validated by quantitative RT-PCR, confirming the high reliability of the RNA-Seq. Our data therefore will not only provide novel insights into the molecular mechanisms that drive the osteogenic differentiation of DPSCs, but also promote the studies of bone tissue engineering that utilizes DPSCs as a crucial resource.

1. Introduction

Dental pulp stem cells (DPSCs) are highly proliferative cells in adults that remain the capability of self-renewal and multi-lineage differentiation [1,2]. Under different conditions, DPSCs may differentiate towards multiple lineages including odontoblast, osteoblasts, adipose cells, neurons, and myocytes [1–3]. The relative ease of isolating DPSCs from discarded or removed teeth makes it convenient to further manipulate these cells in tissue engineering. For example, DPSCs are widely used in dental pulp regeneration and tooth reconstruction [4]. Moreover, DPSCs could also be used in the field of bone tissue engineering after being converted to bone cells [5–7].

In a previous study, we demonstrated that TNF-α promoted the osteogenic differentiation of DPSCs by activating the NF-κB pathway [5]. TNF-α and NF-κB have been shown as important players in osteogenic differentiation *in vitro* [8,9]. However, the underlying mechanisms, especially how different signaling pathways were actively involved during the differentiation of DPSCs, remain unclear. By analyzing the gene expression profiles during TNF-α promoted osteogenic differentiation, we found transitions of gene expression profile at early and later stages of DPSCs differentiation. These findings suggested molecular mechanisms of TNF-α promoted osteogenic differentiation from DPSCs. A better understanding of *in vitro* induced differentiation of DPSCs, may promote the potential usage of autologous transplantation of DPSCs.

2. Materials and methods

2.1. Cell culture

All experiments were approved by the Ethics Committee of the Affiliated Hospital of Nantong University. Normal human impacted third molars were collected from patients with no carious lesions and oral infection. Fresh isolated tooth were cleaned and opened to reveal the pulp chamber. A solution with 3 mg/mL collagenase type I was used to digest pulp cells at 37 °C for 1 h. Single cell suspensions were obtained and cultured with Dulbecco modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum

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(FBS), 100 u/mL penicillin, and 100 μg/mL streptomycin at 37 °C with supplement of 5% CO_2.

2.2. Osteogenic differentiation

Fresh DPSCs were cultured for 3 passages, before being used in the differentiation assay. 2×10^4 cells/cm^2 were cultured in proliferation medium supplemented with 0.1 μM of dexamethasone, 10 mM of β-glycerophosphate (Sigma), 50 mg/mL of ascorbic acid (Sigma) and 10 ng/mL of TNF-a. DPSCs were differentiated for 7 or 14 days before harvest for ALP staining or RNA extraction.

2.3. ALP staining

Undifferentiated DPSCs and DPSCs differentiated for 7 or 14 days were fixed and stained with the ALP assay kit (JianCheng, Nanjing, China) according to the manufacturer's instructions.

2.4. RNA extraction, RNA-seq, and bioinformatic analysis

Total cellular RNA was isolated from cells with the RNeasy plus mini kit (Qiagen). RNA samples were sequenced with a HiSeq 2000. The raw sequencing data was first evaluated by FAST-QC, then analyzed by using MapSplice and EBSeq based on Bowtie (Supplemental Table 1). Genes with fold change equal or higher than 2 or equal or lower than 0.5, and FDR less than 0.05 were considered as different expressed genes. Fisher's exact test was used for Gene Ontology analysis, pathway enrichment analyses, and gene-act-network analysis. A FDR <0.05 was considered as significant.

2.5. Reverse transcription and quantitative PCR

Reverse transcription was performed with the High-capacity cDNA reverse transcription kit (Applied Biosystems). Real-time PCR was performed in triplicate using the QuantiTect SYBR green PCR master mix (Qiagen) and the CFX96 (bi-Rad) qPCR system. The levels of GAPDH mRNA abundance were used for normalization. Primers used for validation were listed in Supplemental Table 2.

3. Results

3.1. TNF-α promoted osteogenic differentiation of DPSCs

DPSCs were treated with osteogenic differentiation medium plus 10 ng/mL TNF-α for 7 or 14 days. After 7 days of TNF-α treatment, DPSCs showed strong ALP activity (Fig 1A), a widely used marker of osteogenic differentiation [10], indicating the DPSCs were undergoing osteogenic differentiation. After 14 days treatment, ALP positive DPSCs increased to 85–90% (Fig 1A), suggesting a nearly complete differentiation of DPSCs.

3.2. Gene expression profiles of differentiated DPSCs

To identify both early and later transcriptional events of TNF-α promoted DPSC differentiation, we harvested DPSCs before, 7 days or 14 days post TNF-α induction, isolated totally RNA, and performed gene expression profiling by next-generating sequencing analysis. There were 1405 genes expressed differentially between undifferentiated and 7 days post TNF-α induction (Fig. 1B), with the cutoff at FDR<0.05 and fold change>2. Among them, 1197 genes were significantly up-regulated, while 208 were down-regulated. After 14 days post TNF-α induction, the number of up-regulated genes slightly increased to 1342, while the number of down-regulated genes was 467 (Fig. 1C). These results indicated that the majority of differentiation required genes were activated/inactivated at early stage of differentiation, yet expression changes of a small subset of genes were specifically involved in the late phase during differentiation.

3.3. Altered gene pathways in differentiated DPSCs

We then performed pathway analysis based on the different expressed genes at day 7 and day 14, respectively. The most enriched pathways at day 7 post TNF-α induction included ubiquitin mediated proteolysis, RNA degradation, ribosomal biogenesis and protein processing in ER (Fig. 2), indicating that global protein and mRNA degradation and concomitant new protein synthesis occurred during the early phase. Such phenomena were also shown in the osteogenic differentiation of mesenchymal stem cells derived from bone marrow (BM-MSCs) [11]. MAPK, and mTOR signaling pathways were also activated. Meanwhile, the TNF-α, along with NF-κB signaling pathway, a downstream activator of TNF-α, was significantly increased at 7 days post TNF-α induction, consistent with our previous results [5].

At 14 days post TNF-α induction, instead of general pathways that mediating protein and mRNA degradation and synthesis at 7 days post induction, specific signaling pathways, including TGF-β, PI3K-Akt were highlighted. These results were consistent with the idea that TGF- β and PI3K-Akt pathways play key roles in osteoblast differentiation [12–14].

3.4. Identification of key genes at different phases during TNF-α promoted osteogenic differentiation of DPSCs

To identify key genes in TNF-α promoted DPSC differentiation, we performed gene regulatory network analysis based on different expressed genes from RNA-seq analyses. We found that genes involved in different signal transduction pathways were in the center of the gene regulatory networks at 7 and 14 days post TNF-α induction, respectively. For example, NRAS, KRAS, PIK3R1, and PIK3CA, which represented MAPK and up-stream of PI3K/PTEN signaling pathways, had the most regulatory interactions with different expressed genes at Day 7 (Fig. 3A, Supplemental Table 3). After 14 days of differentiation, although genes representing MAPK signaling pathway (RAS) were still activated, genes such as ATF2 (TGF-β), and Akt, TP53, two downstream targets of the PIK3 pathway, centered at the pathway interaction network (Fig. 3B, Supplemental Table 3).

To validate the results from RNA-seq and gene regulatory network analysis, we performed quantitative PCR analysis of genes that were highlighted by the gene regulatory network analysis at different time points post TNF-α induction. Consistent with the RNA-seq data, the expression of selected genes showed significant fold changes by quantitative RT-PCR analysis at both time points upon TNF- induction (Fig. 3C,D). Overall, the quantitative RT-PCR data and the RNA-seq results showed high coefficient, indicating the RNA-seq results were highly reliable. (Fig 3C, D).

4. Discussion

DPSCs have been considered as a promising source of cells for regenerative medicine, especially for osteogenic tissues. One of the most critical steps for these applications is to achieve highly efficient and well-controlled differentiation *in vitro*, of which the mechanisms have not been fully understood.

Our results clearly showed a general to specific transformation of gene expression changes during osteogenic differentiation of DPSCs. At the early stage, genes responsible for protein/RNA degradation and synthesis were involved, suggesting a dynamic