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Full-length Transcriptomic Analysis Revealed Regenetative Leydig Cells Come from Different Stem Cell Populations in Testis

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Abstract

Leydig cells (LCs), localized in the interstitial compartment of the testis, produce more than 90% testosterone in male. Our previous study indicated that there were no significant differences in steroidogenic genes expression and androgen production between the original and regenerated LC lineages in testes. After comparison of their Full-length transcriptomic profiles, there were no significant differences in the expression level of most steroidogenic genes. However, the global gene expression pattern bewteen the original and regenerated LC lineages is distinctly different. Moreover, more than 216 genes, including LC lineage marker Nr5a1, have different alternative splicing transcripts. These results suggested that the regenerated Leydig cells and the original Leydig cells may originate from different stem cells.

Keywords

Leydig Cell Lineage; Full-length Transcript Sequencing; Stem Cells; Development.

1. Introduction

Leydig cells are the testicular cells responsible for testosterone production [1]. It is well established that there are dramatic decreases in Leydig cell steroidogenic function that occur with age and/or metabolic-related diseases, with only infrequent turnover of these cells during the lifetime of an animal [2-4]. Previous studies have shown that when Leydig cells are eliminated from the testes of either young adult or aged rats treated by ethane dimethanesulphonate (EDS)[5, 6], regeneration occurs. The developmental process of these regenerated Leydig cells also goes through progenitor, immature, and adult Leydig cell stages[1, 5, 7]. Interestingly, whether the Leydig cells are restored to the testes of young or old rats, they produce testosterone at the high levels of young adult cells[8-10], suggesting that there are Leydig cell precursor cells present in the testes of both young and old rats that are capable of giving rise to well-functioning adult cells[11, 12]. Based on the single-cell sequencing, we suggested that there may not be a specific Leydig stem cell population for Leydig cell generation, but rather that the precursors for ALC regeneration may belong to a broad mesenchymal stromal population[13]. To address this hypothesis, we conducted the Full-length transcript sequencing and analyzed the differences, following the isolation of the preexisting adult Leydig cells (ALCs) and regenerative adult Leydig cells (RALCs) from normal and type 2 diabetic male rats.

2. Methods

2.1. Animals

Sprague–Dawley male rats (postnatal 56d and 90d) were purchased from the Experimental Animal Centre of Guangdong Province (Guangzhou, China). The rats were housed in the animal

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facilities of the School of Medicine under conditions of controlled light (12 h light, 12 h dark) and with free access to food and water. The animal protocol was approved by the Institutional Animal Care and Use Committee of Jinan University.

2.2. Construction of Type-2 Diabetic (T2D) Rat Model

The streptozotocin-induced T2D rat were constructed according to the protocol described previously. Briefly, the SD male rats (postnatal 56 d, 200-250g/each) were fed high-fat diet (22% fat) for a month, followed by one injection streptozotocin (STZ) i.p. at 35 mg/kg dose. After one week, the rats with fast blood glucose (FBG) < 7.8 mmol/L were injected STZ one more time (30 mg/kg). Three weeks after the second injection, the rats with fasting blood glucose of > 16 mmol/L were used for the experiments.

2.3. EDS Treatment

In total, 8 normal male rats (postnatal 90d) and 8 STZ-treated diabetic rats were conducted EDS treatment. To eliminate Leydig cells from the testes, rats were injected with a single dose of EDS (i.p. 75 mg/kg body weight) dissolved in a mixture of DMSO:PBS (1:3). Rats were housed in the animal facilities with free access to water and rat chow. All ALCs were eliminated by 1-week post EDS, and regenerative ALCs formed by 8 weeks. This experiment was repeated 2 times.

2.4. Isolation of Primary Leydig Cells

For the isolation of primary LCs, four groups of rats, including wildtype control group (WC), diabetic model group (DC), EDS-treated wildtype group (WE) and EDS-treated diabetic group (DE), were used. The testes were enzymatically dispersed with 0.25 mg/ml collagenase D in Medium 199 for 10 min at 34°C. The dispersed cells were filtered through two layers of 100-μm pore size nylon mesh, centrifuged at 250×g for 10 min, and re-suspended in 55% isotonic Percoll to separate cells by buoyant density. After centrifugation at 23,500×g for 45 min at 4°C, the fraction of LCs with densities between 1.068 and 1.070 g/ml was collected. The purity of isolated Leydig cells was more than 95% according to the 3β-Hydroxysteroid dehydrogenase (3βHSD) activity staining.

2.5. Differential Expression Genes Detection and Alternative Splicing Analysis

Total RNA of each sample was extracted according to the instruction manual of the TRlzol reagent and the mRNA was isolated subsequently. The quality and quantity of mRNA was assessed using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Libraries of direct RNA sequencing were constructed following the ONT Direct RNA Sequencing method. The reads were filtered with a minimum average q-score of 7 and the length <50 bp. Gene level counts were produced for each nanopore replicate using feature Counts version 1.6.3 in long-read mode with strand-specific counting. DESeq and the FDR-adjusted *p*-value (False discovery rate) were employed and used to evaluate differential expression genes (DEGs) based on the ratio of the fragments per kilobase of exon per million fragments mapped (FPKM) values. The DEGs between samples were identified with fold change value ≥ 2 or ≤ 0.5, and *p*-value < 0.01, that were then divided into up- and down-expression groups. The enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and GO terms were performed by METASCAPE (version 3.5, https://metascape.org/) and DAVID software (Database for Annotation, Visualization and Integrated Discovery, version 6.8, http://david.abcc.ncifcrf.gov/) respectively.

In order to simplify and visualize multidimensional patterns, multidimensional scaling (MDS) analysis was performed using the edgeR function plotMDS with pair-wise log fold-changes estimated on read counts (normalized to library size) for the 250 most heterogeneously

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expressed genes. As for the Alternative Splicing Analysis, the SUPPA2 method was used to analyze the alternative splicing of each individual (https://github.com/comprna/SUPPA).

2.6. Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

For mRNA qRT-PCR, the total RNAs from the cells and tissues were extracted and used as templates for cDNA synthesis. All PCRs were performed using a Bio-Rad CFX Connect Real-time system (Bio-Rad Laboratories, CA, USA), and the data were collected using the Bio-Rad CFX Manager software (version 2.0). The relative expression levels of the targeted mRNAs were normalized against the expression of *rps16*. The fold changes of the expression between the treatments and controls were calculated by the $2^{-\Delta\Delta Ct}$ method. The efficiency of qRT-PCR performance for target genes is between 100-105%. All data were derived from three different independent experiments.

2.7. Testosterone Analysis by Radioimmunoassay

For the analysis of testosterone concentration, the serum from DE, WE and WC were collected and measured using RIAs. Briefly, the standards, controls, and samples (50 µl in duplicate) were dispensed into numbered tubes. Subsequently, 100 µl ^{125}I-labeled androgen tracer and 100 µl primary antibody were added to the appropriate tubes. The tubes were shaken for 10 s and incubated in a water bath for 1 h at 37°C. Then, 500 µl of secondary antibody was added to all tubes (except the total-count tubes) and incubated for 15 min at room temperature. The tubes were then centrifuged at 1800×g for 15 min at 4°C. The supernatants were decanted, and the radioactivity in the precipitate was quantified for 1 min. The sensitivity of this assay system was 0.01 µg/l. The intra-assay and inter-assay variations were less than 10% and 15%, respectively. The results of four separate experiments were averaged for statistical analysis.

2.8. Statistical Analysis

The data were analyzed by one-way ANOVA, followed by Tukey's ad hoc multiple comparison tests. All data are expressed as the mean±S.E.M. Differences were regarded as significant at $p<0.05$.

3. Results

3.1. The Expression Analysis of Steroidogenic Genes

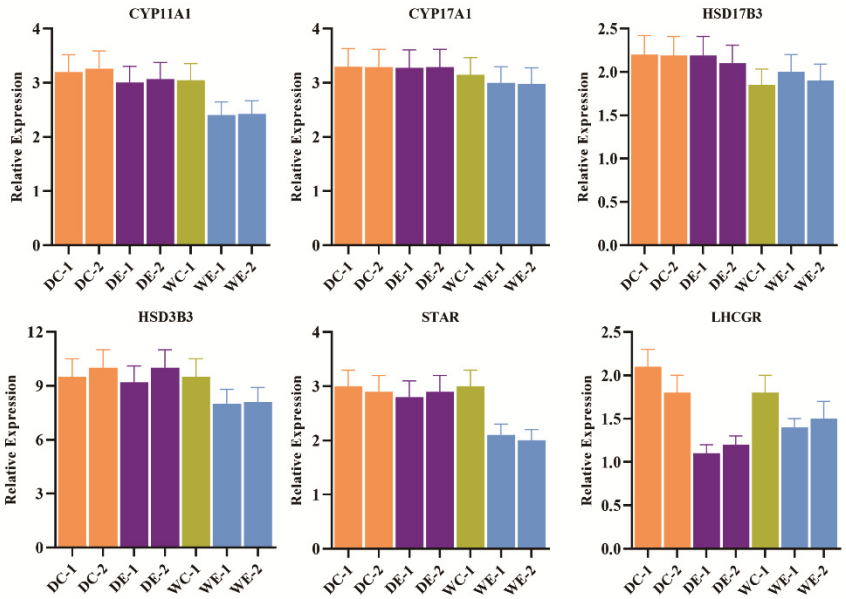

Figure 1. The expression level of steroidogenic genes in different groups

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The RT-PCR result showed that the expression level of steroidogenic genes in DC group was similar with that of WC group. As for the regenerative Leydig cells, only the expression of Lhcgr in DE group and Star in WE group slightly decreased compared with the control (Figure 1). We further analyzed the concentration of testosterone. As expected, the result indicated that the concentration of testosterone among DE, WE and WC groups was similar (Figure 2).

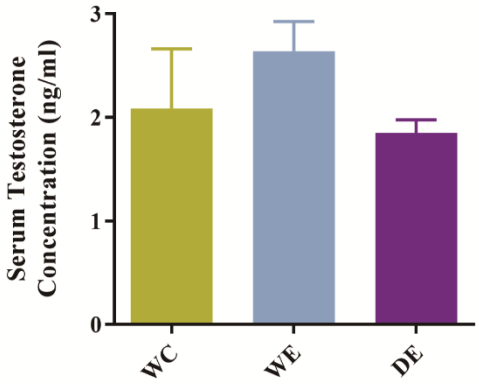

Figure 2. The testosterone production analysis in DE, WC and WE groups

3.2. Similarity Analysis of Transcriptomic Profiles

To assess the similarity level of individual groups, the multidimensional scaling (MDS) analysis was conducted. The datasets from WC, WE, DC and DE were clustered individually, and they were apart from other datasets spatially (Figure 3), suggesting that the profiles derived from the same treatment had the bigger similarity than from others.

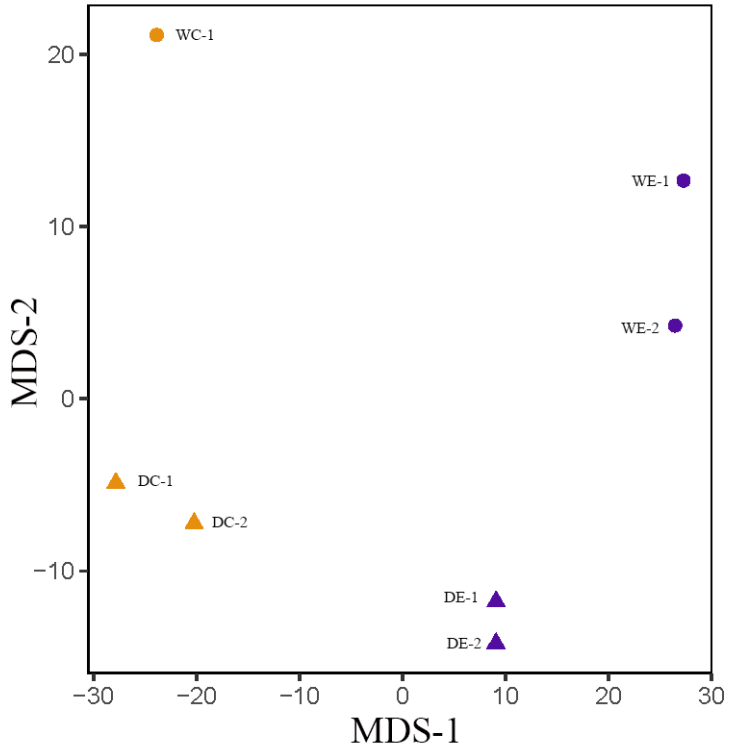

Figure 3. Multidimensional scaling (MDS) analysis between different groups

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3.3. Differentially Expressed Analysis of Transcriptomic Profiles

A total of 32,884 genes were detected in each treatment group. There were only 1,081 differentially expressed genes (DEGs, foldchange > 2 and p value < 0.05) between WC and DC. These genes mainly enriched in KEGG pathways including the GnRH, MAPK and Rap1 signaling pathways (Figure 4A). In terms of the comparison of WC and WE groups, 2,520 DEGs were enriched in IL-27-mediated, antigen receptor-mediated, and immune response-regulating signaling pathways (Figure 4B). In total of 1,972 DEGs between DC and DE were enriched in T cell receptor signaling pathway, PPAR signaling pathway, cell cycle, insulin resistance and apoptosis (Figure 4C). In DE and WE comparison, 2,476 DEGs were identified and enriched in carbon metabolism, pentose phosphate pathway, glycerophospholipid metabolism and cholesterol metabolism. A 2-way hierarchical clustering analysis shown that the DEGs between DE and WE are much similar to each other than to WC and DC (Figure 5A).

The transcription factor (TF) expression pattern reflect cell fate, therefore, we next investigated the tendency of TFs expression among WC, DC, WE and DE. All TFs, in total 1000 genes, were clustered and conducted the pairwise comparison. The hierarchical clustering analysis further confirmed that the TF expression tendency of DE was close to that of WE (Figure 5B).

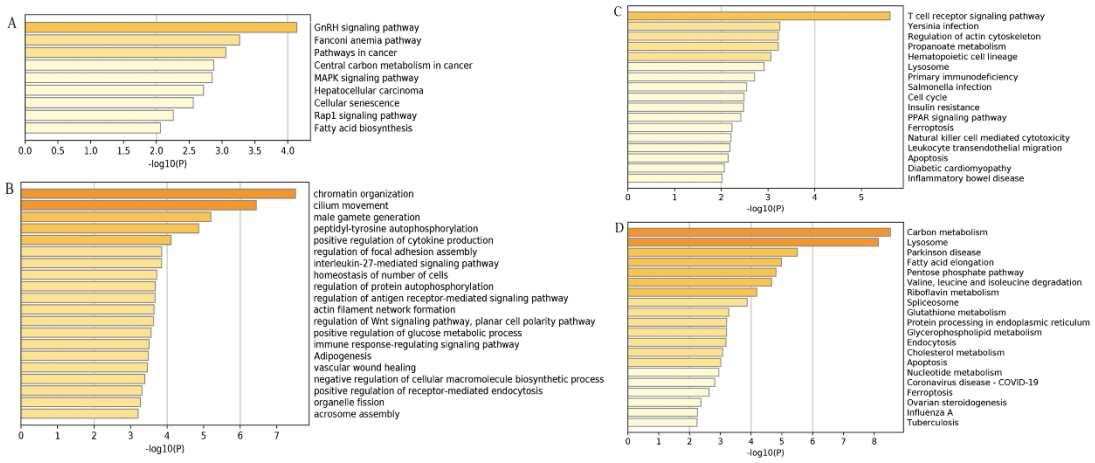

Figure 4. KEGG pathway enrich analysis between different groups

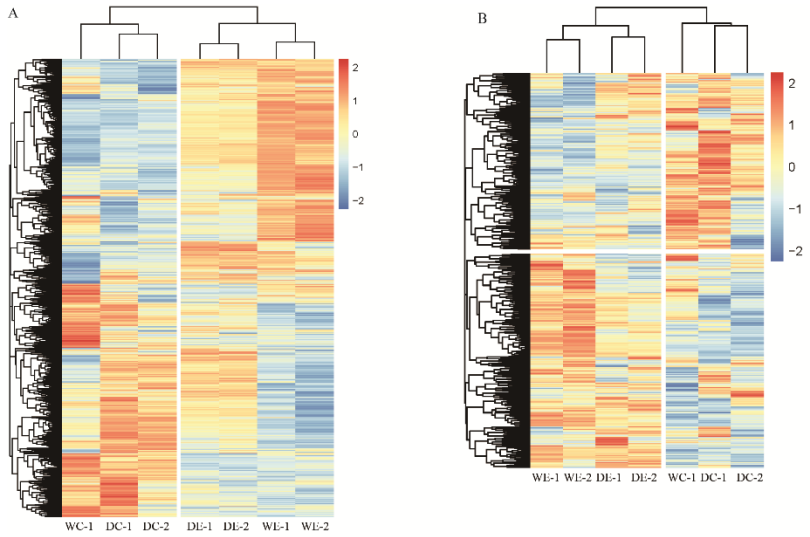

Figure 5. DEGs analysis (A) and transcription factor (B) expression analysis between different groups

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3.4. Alternative Splicing Analysis

Alternative splicing is an important transcriptional regulatory mechanism for gene regulation and for generating cellular protein diversity. We investigated the differences of alternative splicing between wildtype and regenerative LCs based on the Full-length transcriptomic sequencing data. In total of 216 genes, including insulin receptor (INSR), NR5A1 and Slc38a1, were identified. The LCs from WE group and the LCs from DE did not express the isoform 2 of Nr5a1 and the isoform 2 of INSR (Figure 6).

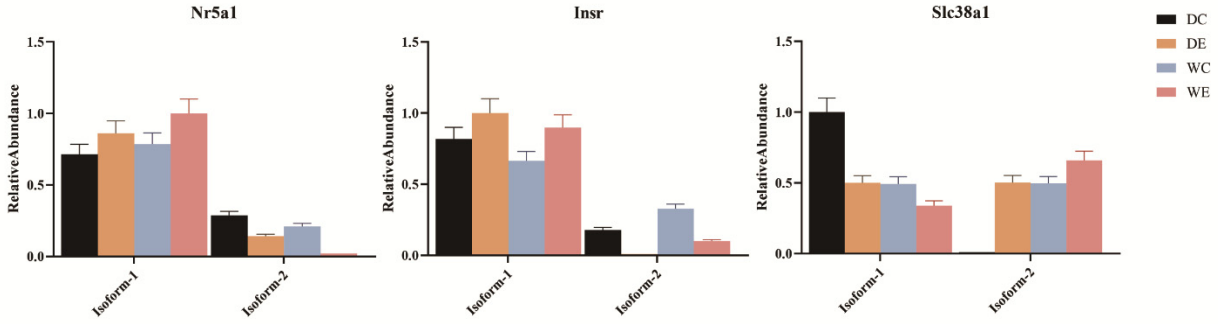

Figure 6. The differences of alternative splicing between different groups

4. Discussion

It is well demonstrated that after the elimination of original LC lineage by the administration of EDS, a new LC population regenerated. The regenerative LCs have a powerful capacity of synthesize androgens, which restore the testosterone concentration to normal level in hypogonadal animals. In this study, we compared the Full-length transcriptomic sequencing data from original and regenerative LCs of normal and diabetic models. The significant differences between original and regenerative LC demonstrated that there is a number of precursor cells belonging to different stem cell populations that can form LCs in the adult testis. Importantly, the niche of testis interstitium is essential for the LC lineage formation from stem cells.

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