BET bromodomain inhibitor JQ1 regulates spermatid development by changing chromatin conformation in mouse spermatogenesis

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Abstract
As a BET bromodomain inhibitor, JQ1 has been proven to have efficacy against a number of different cancers. In terms of male reproduction, JQ1 may be used as a new type of contraceptive, since JQ1 treatment in male mice could lead to germ cell defects and a decrease of sperm motility, moreover, this effect is reversible. However, the mechanism of JQ1 acting on gene regulation in spermatogenesis remains unclear. Here, we performed single-cell RNA sequencing (scRNA-seq) on mouse testes treated with JQ1 or vehicle control to determine the transcriptional regulatory function of JQ1 in spermatogenesis at the single cell resolution. We confirmed that JQ1 treatment could increase the numbers of somatic cells and spermatocytes and decrease the numbers of spermatid cells. Gene Ontology (GO) analysis demonstrated that differentially expressed genes which were down-regulated after JQ1 injection were mainly enriched in “DNA conformation change” biological process in early developmental germ cells and “spermatid development” biological process in spermatid cells. ATAC-seq data

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Introduction

Mammalian spermatogenesis is a complex, elaborate and orderly process, which mainly involves three stages: the proliferation and differentiation of spermatogonia via mitosis, the production of haploid spermatids from primary spermatocytes via meiosis and spermiogenesis.\(^1\)\(^2\) The successful completion of spermatogenesis requires the coordinated interaction of multiple genes, and their expression is precisely controlled in time and space.\(^3\)

In mammals, the bromodomain and extra-terminal (BET) family is composed of BRD2, BRD3, BRD4 and BRDT. These four proteins are all involved in spermatogenesis, and BRDT is exclusively expressed in the testis.\(^4\) BRD4 initiates a programming guided by histone acetylation in meiotic and post-meiotic cells and directs the replacement of histones by transition proteins in post-meiotic cells.\(^5\)\(^6\) BRD4 binds to the acetylated tails of histones H3 and H4 and occupies thousands of enhancers related to active genes and a small set of exceptionally large super-enhancers related to genes that feature prominently.\(^7\)\(^8\)\(^9\)\(^10\)\(^11\)\(^12\) Studies have confirmed that super-enhancers are involved in the control of mammalian cell identity in health and in disease.\(^13\)\(^14\)\(^15\)\(^16\)

Inhibition of BRD4 by BET bromodomain inhibitors, such as JQ1, could affect the expression of super-enhancer associated genes.\(^17\) Furthermore, JQ1 binds competitively to the ε-N-acetylation of lysine residues binding site of BET family bromodomains and dramatically increases the thermal stability of all bromodomains of the BET family, but does not affect the stability of other bromodomains outside the BET family.\(^18\) In vivo studies have demonstrated that intraperitoneal (ip) injection of JQ1 in male mice could result in impaired spermatogenesis and reduced motility. In particular, this is reversible, which means that JQ1 may be used as a potential contraceptive.\(^19\) However, the specific mechanism of JQ1 acting on spermatogenesis and the side effects after drug treatment are still unclear.

Here, by combining single-cell RNA-seq and ATAC-seq, we demonstrated that JQ1 injection would increase the numbers of somatic cells and spermatocytes and decrease the numbers of spermaticid cells. Moreover, JQ1 could modulate the expressions genes related to spermatid development by changing the chromatin conformation of germ cells in the early developmental stage to achieve the purpose of regulating spermiogenesis and sperm maturation.

Materials and methods

Experimental animals

C57BL/6 mice were purchased from Vital River Laboratories (Beijing, China) and housed in the Animal Center of Nantong University under a 12-h light/dark cycle at a temperature of 23 ± 2 °C and humidity of 50%–70%. All animal experiments were approved and conducted in compliance with the Nantong University Animal Care and Use Committee.

JQ1 and control treatment

The dosages and injection method of JQ1 and the control were based on Matzuk’s description.\(^19\) Specifically, (+)-JQ1 (ApexBio, Houston, USA; A1910) was dissolved in DMSO at 50 mg/ml and then diluted 1:10 in 10% 2-hydroxypropyl-b-cyclodextrin (Sigma, California, USA; 332607-25G), which was referred to as JQ1 in this study. JQ1 was ip injected into male mice at 1% of the body weight (50 mg/kg). The control was DMSO dissolved 1:10 in 10% 2-hydroxypropyl-b-cyclodextrin and ip injected into male mice at 1% of the body weight. Juvenile (3 weeks old) C57BL/6 male mice in these studies were weighed daily before injections.

Hematoxylin and eosin staining

Testicular tissues of WT, control and JQ1-treated mice were dissected out and fixed with 4% paraformaldehyde overnight, then embedded in paraffin wax and sectioned at 4 mm. For histological evaluation, sections were stained with hematoxylin and eosin (Sangon Biotech, Shanghai, China; E607318-0200) using standard techniques for histological evaluation.

Tissue dissociation and single-cell suspension preparation

The preparation method of single cell suspension of testis is as described in Luo’s description.\(^20\) Specifically, testes of WT, control and JQ1-treated group were removed and minced by scissors, then incubated in 10 ml DMEM (Life Technologies, NY, USA; 11995-081) containing 1 mg/ml collagenase IV (Sigma, California, USA; C5138-1G) and 1 unit/ml DNase I (Sigma, California, USA; AMDP1-1KT) in a
shaking water bath at 32 °C for 10 min. The seminiferous tubules were collected by centrifugation at 500g for 2 min. The pellet was washed once with DMEM and re-suspended in 10 ml DMEM containing 1 mg/ml Trypsin (Sigma, California, USA; T1426-500MG) and 1 unit/ml DNase I (Sigma, California, USA; AMPD1-1KT), then incubated in a shaking water bath at 32 °C for 10 min. Cells were collected by centrifugation at 500g for 2 min. The cell pellet was washed twice with DMEM and filtered through a 40 μm Nylon Cell Strainer (BD Falcon, NJ, USA; 352340) to separate cells from cell debris and other impurities. The cells were centrifuged at 1000 rpm for 5 min at 4 °C and cell pellets were re-suspended in 1ml PBS (Cytiva, MA, USA; SH30258.02). To remove red blood cells, 2 mL GEXSCOPETM Red Blood Cell Lysis Buffer (Singleron Biotechnologies, Nanjing, China) was added and incubated at 25 °C for 10 min. The mixture was then centrifuged at 1000 rpm for 5 min and cell pellet was re-suspended in PBS. Cell numbers were obtained by TC20 automated cell counter (Bio-Rad, California, USA).

Library preparation of scRNA-seq

scRNA-seq was performed on testes from WT, control and JQ1-treated mice by using the Singlelon GEXSCOPETM technique (Singleron Biotechnologies, Nanjing, China) following the protocol in Dura’s description.21 Each experimental group has only one sample. Briefly, the concentration of single-cell suspension was adjusted to $1 \times 10^5$ cells/mL in PBS (Cytiva, MA, USA; SH30258.02) and loaded onto a microfluidic chip (part of Singleron GEXSCOPETM Single Cell RNAseq Kit, Singleron Biotechnologies, Nanjing, China). scRNA-seq libraries were constructed according to manufacturer’s instructions (Singleron Biotechnologies, Nanjing, China) and sequenced on Illumina HiSeq X Ten at Novogene (Beijing, China) with 150 bp paired end reads.

Data analysis of scRNA-Seq

Based on library preparation and sequencing of scRNA-seq above, each sample obtained two paired-end reads. Read 2 was separated by specific cell barcode information, and read 1 had alignment information based on Unique Molecular Identifier (UMI).22 UMI-tools (v1.0.1) was used to align the fastq reads which contained UMs ± cell barcodes, remove UMI sequences from fastq reads and appende UMls to the read name. The processed data were mapped to the mouse reference genome (GRCm38.p6, Gencode) through STAR (v2.7.5a).23 The gene expression data of each cell for the forward strand was calculated and identified by their morphological characterization and Q-PCR with germ cell type-specific marker genes (Sycp3 for pacSC, and Prm2 and Tnp1 for RS). The Seurat package (v3.1.0)23 in R was used to screen cells according to the following three criteria: the number of genes detected in a single cell, the number of UMls and the percentage of reads that map to the mitochondrial genome.26 We filtered cells that have unique feature counts over 6000 or less than 1000 and mitochondrial counts >10%. The SCTransform function was performed to normalize and scale the data. The principal component analysis was performed on the scaled data to separate the cells. The PCs 1–30 were selected to perform the RunTSNE function27 or RunUMAP function,28 the FindNeighbors function and the FindClusters function (resolution = 0.8) to obtain the cell clusters. The FindAllMarkers function was used to identify unique cluster-specific marker genes. We chose the genes with the value of p_val_adj < 0.05 and performed GO analysis using the clusterProfiler (v.3.16.0)29 and org.Mm.meg.db (v3.11.1) package. Through the results about the cell-specific genes and the GO function enrichment analysis about cluster-specific marker genes, we determined the cell type of each cell cluster. Then we used jupyter notebook (v1.0.0) to show the results about the expression of cell-specific genes in different cell types.

Based on the highly variable genes obtained from Seurat, Monocle 3 was conducted to generate the pseudo-time trajectory for the different stages of germ cells.30,31 We adopted the following three steps: choosing genes that define the progress of a cell, reducing data dimensionality and ordering cells along the trajectory to construct single-cell trajectories. The plot_cell_trajectory function was used to show the lines plots of pseudotime order with fitted curves of all the samples.

The function subset in Seurat package was used to obtain the cell numbers of each cell type. FindMarkers function in Seurat package was used to identify differentially expressed genes (DEGs) between different samples on the same cell types. DEGs were defined only if the adjusted $P$ values and FDR were both $<0.05$, and a fold change (log2-transferred) was $>1$ or $<-1$ which presented as up-regulated and down-regulated genes respectively. According to the DEGs, we performed GO function enrichment analysis to find biological functions or pathways that are significantly related to the genes specifically expressed.

Purification of pacSC and RS from mouse testes

pacSC and RS from WT, control and JQ1-treated mouse testes were isolated using STA-PUT method based on Bellvé’s description with minor modifications. After obtaining the single-cell suspension, cells were collected by centrifugation at 500g for 2 min and re-suspended in 40 ml DMEM (Life Technologies, NY, USA; 11995-081) containing 0.5% BSA (Sangon Biotech, Shanghai, China; AD0023-100 g). Then the cells were separated by sedimentation velocity at unit gravity at 4 °C, using 2–4% BSA gradient in DMEM. Only fractions of the expected cell type and purity ($\geq 75\%$) were pooled together. The purities of pacSC and RS were evaluated and identified by their morphological characterization and Q-PCR with germ cell type-specific marker genes (Sycp3 for pacSC, and Prm2 and Tnp1 for RS).
expression. Q-PCR primer sequences were listed in Table S2 (online).

Library preparation ATAC-seq

ATAC-seq was applied essentially following our previous description and performed on pacSC and RS from WT, control and JQ1-treated mice, respectively. These six cell samples all had two replicates. The libraries were sequenced on Illumina HiSeq X Ten and NovaSeq 6000 at Novogene (Beijing, China) with 150 bp paired end reads.

Data analysis of ATAC-seq

After library preparation and sequencing of ATAC-seq, we got raw paired-end reads. Firstly, the software fastp (v.0.20.1) was used for quality control and filtering the raw reads. Secondly, all reads for each sample were combined and aligned to mm10 (UCSC version) with bowtie2 (v.2.4.1). Thirdly, macs2 (v2.2.7.1) was used to find peaks for each sample and deeptools (v3.4.3) was applied to check the signal strength of the gene body (such as TSS and TES). Then Homer (v4.11) was chosen to annotate the peaks and count peak distributions in promoter, exon, intron and intergenic regions. The transcriptional peak regions were identified using DiffBind (v2.16.0) based on statistical model DESeq2. Homer (v4.11) was used to search and annotate the motifs in the differential peak regions. The transcript ID of the differential peak regions was converted to gene ID on David (v6.8). In addition, GO and KEGG analysis was also performed on David (v6.8). The list of meiotic DNA double strand break sites for the peaks was generated from previously published DMC1 ChIP-seq. Availability of data

The raw sequencing data (scRNA-seq and ATAC-seq) reported in this paper are available in the Genome Sequence Archive in the Beijing Institute of Genomics data center: CRA002896.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism to assess differences between experimental groups. Statistical significance was analysed by Student’s t-test and expressed as a P-value. P-values lower than 0.05 were considered to be statistically significant. * P < 0.05; ** P < 0.01; *** P < 0.001.

Results

Inhibition of BET bromodomain by JQ1 triggers male germ cell defects in mouse

To assess the possible effect of inhibition of super enhancer in spermatogenesis, 3 weeks juvenile C57BL/6 male mice were administered JQ1 (the mixture of (+)-JQ1, DMSO and 2-hydroxypropyl-β-cyclodextrin) (50 mg/kg once daily) or vehicle control (the mixture of DMSO and 2-hydroxypropyl-β-cyclodextrin) via ip injection daily for 3 weeks, and the records of mouse daily weight were shown in Figure S1A. After the treatment, mice were sacrificed. Compared with control and WT mice (wild type mice of the same age without any treatment), JQ1-treated mice revealed a significant reduction in testicular volume (Fig. 1A). In terms of the testis weight, JQ1-treated mice showed a reduction to 68% of control and 67% of WT mice (Fig. 1B).

To examine the effect of JQ1 on various types of cells in the testis from a histological level, we performed hematoxylin and eosin staining, and the results demonstrated a reduction in the numbers of round spermatids and subsequent types of germ cells after JQ1 treatment, and some abnormal spermatids appeared to have large nuclei and abundant cytoplasm and symplasts, as indicated by black arrow (Fig. 1C), which was consistent with previous study. These results suggest that the BET bromodomain inhibitor JQ1 could cause germ cell defects and affect testicular development in male mice.

Single-cell transcriptome profiling and cell type identification of JQ1-treated mouse testes

To investigate the cell-type-specific alterations in gene expression after JQ1 or control treatment at single-cell resolution, we performed single-cell RNA sequencing (scRNA-seq) on testes from WT, control and JQ1-treated mice by using the Singleon GEXSCOPE™ technique (Singleron Biotechnologies, Nanjing, China) following the protocol in Dura’s description (Fig. S2A). After obtaining high-throughput sequencing data of scRNA-seq, we first filtered the data, and screened out the cells with the number of feature RNA between 1000 and 6000 and the percentage of mitochondrial genes below 10% for further analysis.
JQ1 regulates spermatid development in mouse spermatogenesis

| Page 1067 |

Global patterns of single-cell transcriptome profiling and cell type identification. (A) UMAP plots showing 22 clusters of testicular cells. Left: Control mouse; Middle: JQ1 mouse; Right: WT mouse. (B) UMAP plots showing 19 clusters of germ cells and 3 clusters of somatic cells. Left: Control mouse; Middle: JQ1-treated mouse; Right: WT mouse. (C) Violin plots showing the expression levels of cell type marker genes based on RNA-seq analysis throughout 19 clusters of germ cells and 3 clusters of somatic cells. (D–E) Developmental pseudotime of male germ cells of WT mouse.
Changes in DNA conformation in JQ1-treated mouse testes

To further understand the influence of JQ1 on specific functional pathways in spermatogenesis, we performed GO analysis on the DEGs in the remaining cell clusters between control and JQ1-treated mice. Notably, for genes down-regulated in JQ1-treated mice, "nucleus organization", "DNA packaging" and "DNA conformation change" were the three most frequently occurring biological processes, which occurred not only in germ cells (Diff. SG, SC-1, SC-2, SC-3, SC-4, SC-5, RS-1 and RS-2), but also in somatic cells (Macrophages) (Fig. 4A).

To verify the regulatory function of JQ1 on DNA conformation, we tested the RNA expression levels of some genes correlated with DNA conformation change in testicular cells. The results of real-time fluorescence-based quantitative PCR (Q-PCR) illuminated that the transcription levels of $Prm1$, $Prm2$, $Prm3$, $Tnp1$, $Tnp2$ and $Hils1$ genes were significantly reduced after JQ1 treatment (Fig. 4B). To explore whether this phenomenon also exists in specific germ cell types, such as pacSC and RS, we isolated and purified pacSC and RS from WT, control and JQ1-treated mice by using STA-PUT velocity sedimentation method$^{20,32,45}$ and validated the cell types and purities of these cells by morphological characterization (Fig. S4A) and Q-PCR with cell-type-specific markers ($Sycp3$ for pacSC, and $Prm2$ and $Tnp1$ for RS) (Fig. S4B–D). Results revealed that the differential expressions of genes related to DNA conformation change in isolated cells were more remarkable, especially in RS (Fig. 4C, D). These phenomena suggest that JQ1 treatment may affect the gene expression by changing the chromatin conformation, which in turn causes germ cell defects.

Changes in chromatin accessibility in JQ1-treated mouse testes

The open state of chromatin is one kind of chromatin conformation. To further investigate the effect of JQ1 on the change of chromatin conformation, we conducted assay for transposase accessible chromatin with high-throughput sequencing (ATAC-seq$^{46}$) to measure chromatin accessibility in pacSC and RS in three groups, respectively. First, we found that the distributions of peaks on gene functional elements were different among six different cell samples and the
proportion of the peaks involved in promoter in pacSC was higher than that in RS in the same experimental group (Fig. 5A). Then we found that the intensity of the accessible chromatin signal around transcriptional start sites (TSS) and transcriptional elongation site (TES) decreased as germ cell development, and the intensity in control group was stronger than that in JQ1-treated group, while that in WT group was the weakest (Fig. 5A). TF motif analysis revealed that differentially accessible chromatin regions between control pacSC and JQ1 pacSC and between control RS and JQ1 RS were enriched with same TF motifs, like NFY, KLF1, Sp2, Sp1 and Sp5, although P-values were slightly different (Fig. 5B). Meiotic recombination is initiated by the introduction of DNA double-stranded breaks (DSBs). Thus, we compared the numbers of accessible meiotic DSB sites among six samples, and unexpectedly found that the number of accessible meiotic DSB sites of JQ1 pacSC was lower than that of control pacSC, while the number of accessible meiotic DSB sites of JQ1 RS was slightly higher than that of control RS (Fig. 5C). These results suggested that JQ1 treatment may affect spermatogenesis by regulating meiotic recombination. GO analysis on the differentially accessible chromatin regions between control pacSC and JQ1 pacSC showed that differentially peaks took part in multiple functional pathways related to chromatin conformation, including "DNA repair", "covalent chromatin modification", "organelle fission", "histone modification" and so on (Fig. 5D). KEGG analysis showed that the differentially accessible chromatin regions between control pacSC and JQ1 pacSC were mainly involved in "Protein processing in endoplasmic reticulum", "Huntington disease" and "Parkinson disease" (Fig. 5E). In addition, GO analysis on the differentially accessible chromatin regions between WT pacSC and control pacSC showed that the vehicle control may affects the chromatin

Figure 4  Changes in DNA conformation after JQ1 treatment. (A) First five GO terms of DEGs down-regulated in JQ1-treated group compared to control in nine cell clusters (inducing Diff. SG, SC-1, SC-2, SC-3, SC-4, SC-5, RS-1, RS-2 and Macrophages). (B) The relative expressions of Prm1, Prm2, Prm3, Tnp1, Tnp2 and Hils1 between control and JQ1-treated mouse testes were examined by Q-PCR. (C) The relative expressions of Prm1, Prm2, Prm3, Tnp1, Tnp2 and Hils1 between control pacSC and JQ1 pacSC were examined by Q-PCR. (D) The relative expressions of Prm1, Prm2, Prm3, Tnp1, Tnp2 and Hils1 between control RS and JQ1 RS were examined by Q-PCR. All data in (B–D) were presented as the means ± SEMs from at least three independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 5  Changes in chromatin accessibility after JQ1 treatment. (A) The intensity of the accessible chromatin signal around TSS and TES of pacSC and RS from control, JQ1-treated and WT mice. (B) TF motif analysis on differentially accessible chromatin regions between control pacSC and JQ1 pacSC and between control RS and JQ1 RS. (C) The numbers of accessible meiotic DSB sites among six samples. (D) GO analysis on the differentially accessible chromatin regions between control pacSC and JQ1 pacSC. (E) KEGG pathway analysis on the differentially accessible chromatin regions between control pacSC and JQ1 pacSC. (F) GO analysis on the differentially accessible chromatin regions between WT pacSC and control pacSC. (G) The accessibility of Prm1, Prm2, Prm3 and Tnp2 genes which were located on chromosome 16.
JQ1 regulates spermatid development in mouse spermatogenesis

Discussion

Effective contraception is needed for reproductive health. To achieve the goal of birth control, various methods of regulating fertility must be available. In many societies, men are ready to share the responsibility of contraception more equally with their partners. Therefore, the development of new and effective methods of male contraceptive methods has become a priority.

Previous studies have shown that BET bromodomain inhibitor JQ1 could penetrate the blood-testis barrier and affect sperm motility. Here, we further investigated the effect of JQ1 on gene transcription in each type of germ cell and somatic cell from the single-cell resolved testis. We explored the accessibility of six genes (Prm1, Prm2, Prm3, Tnp1, Tnp2 and Hils1) correlated with DNA conformation change by scRNA-seq data and ATAC-seq technology. We performed ATAC-seq on these cell samples, respectively. ATAC-seq data further demonstrated that treatment of JQ1 could result in changes of chromatin accessibility (Fig. 5). In general, our results revealed that JQ1 regulated gene expression by changing chromatin conformation and is closely related to gene transcription.

In addition, the mixture of DMSO and 2-hydroxypropyl-b-cyclodextrin was extensively used as a vehicle control in biological and medical experiments. But in this study, we found that the mixture had a certain solvent toxicity. The mixture could result in a large number of DEGs appear in SC-6 and ES-1 (Fig. 5B) and affect the chromatin accessibility of genes related to organelle function (Fig. 5F). Therefore, when the mixture is used as vehicle control group, it is necessary to pay attention to the dosage and add an untreated control group to detect the solvent toxicity.

Author contributions

X.W. and S.G. performed experiments with assistance from C.Z., X.Z, G.W., Z.L and Y.H.; M.S. performed data analysis with assistance from X.W., S.C. and G.X.; F.S and E.D. designed the project; X.W., M.S., F.S and E.D. wrote and reviewed the manuscript.

Conflict of interests

The authors declare that they have no conflict of interests.

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Abbreviations

ATAC-seq the assay for transposase accessible chromatin with high-throughput sequencing
BET the bromodomain and extra-terminal domain
Control/vehicle control the mixture of DMSO and 2-hydroxypropyl-b-cyclodextrin
DEGs differentially expressed genes
Diff. SG differentiating spermatogonia
dipSC diplotene spermatocytes
DSB DNA double-stranded break
ES elongating spermatids
GO Gene Ontology
ip intraperitoneal
JQ1 the mixture of (++)-JQ1, DMSO and 2-hydroxypropyl-b-cyclodextrin
pacSC pachytene spermatocytes
Q-PCR real-time fluorescence-based quantitative PCR
RS round spermatids
SC spermatocytes
scRNA-seq Single-cell RNA sequencing
TES transcriptional elongation site
TF transcription factor
t-SNE t-distributed stochastic neighbor embedding
TSS transcriptional start site
UMAP uniform manifold approximation and projection
UMI Unique Molecular Identifier
WT wild type

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jendis.2020.12.012.

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