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Research article

EEF1B2 regulates the proliferation and apoptosis of human spermatogonial stem cell lines through TAF4B

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ABSTRACT

Background: Spermatogonial stem cells (SSCs) are essential for male fertility, maintaining sperm production throughout life. While mouse SSCs have been studied extensively, the mechanisms regulating human SSCs are less understood.
Objectives: To investigate the role of EEF1B2 in regulating human SSC proliferation and apoptosis.
Material and methods: Single cell RNA sequencing (scRNA-seq) analysis was utilized to investigate the differentially expressed genes of SSC. The distribution of EEF1B2 in the human testis was examined using immunofluorescence and immunohistochemistry techniques. Cell proliferation, DNA replication, and self-renewal were analyzed using CCK8, EdU, Western blot, and flow cytometry. RNA sequencing was employed to analyze the downstream target molecules and signaling pathways of EEF1B2.
Results: In this study, we analyzed single-cell sequencing data from human testicular samples and identified EEF1B2 as a protein highly expressed in SSCs, with expression decreasing during development. Immunohistochemistry and immunofluorescence confirmed this pattern and showed co-localization with the proliferation marker KI67. Knockdown of EEF1B2 in human SSC lines impaired proliferation and viability, reducing self-renewal proteins like PLZF and CCNE1. RNA sequencing revealed decreased TAF4B following EEF1B2 knockdown, which could be rescued by replenishing TAF4B. Testicular SSCs from non-obstructive azoospermia (NOA) patients also showed reduced EEF1B2.
Discussion and conclusion: Our findings reveal a novel regulatory mechanism involving EEF1B2 and TAF4B in human SSCs, suggesting EEF1B2 deficiency may contribute to male infertility.

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1. Introduction

Infertility is an escalating worldwide health concern, impacting around 15 % of couples, with male factors being responsible for more than 50 % of instances [1]. Azoospermia, distinguished by the total absence of sperm in the ejaculate, is a severe kind of male infertility, especially when caused by non-obstructive mechanisms (NOA) [2]. NOA is typically categorized into pre-testicular and testicular types, depending on the location of the etiology. Pre-testicular NOA arises due to hormonal abnormalities, while testicular NOA is associated with intrinsic testicular defects that impair spermatogenesis. Based on histological features, NOA can be classified into maturation arrest, hypospermatogenesis, and Sertoli-cell only. Furthermore, mixed histological patterns are often observed in men with NOA [2,3]. NOA is a challenging condition to treat clinically, as it has multiple etiologies, including genetic, endocrine, congenital, drug-induced, chemotherapy-related, and immunological factors [4,5]. NOA may occur due to abnormalities at any point in the process of spermatogenesis, ranging from the first germinal stem cells to fully developed sperm. However, the pathogenesis of NOA remains poorly understood, and the fertility prospects of NOA patients are limited. Therefore, elucidating the molecular and cellular mechanisms of NOA and developing effective therapeutic strategies are important goals for reproductive medicine.

Stem cell regenerative medicine has enabled the recovery of various tissues and organs by using adult stem cells [6]. Spermatogonial stem cells (SSCs) are the source of spermatogenesis, and they maintain adult spermatogenesis by continuously self-renewing and differentiating into sperm [7]. The application of SSCs to correct defective spermatogenesis resulting from germ cell defects has been achieved in mice. In 1994, Brinster et al. successfully restored spermatogenesis in sterile mice by transferring spermatogonia from newborns [8]. In 2000, Meng et al. demonstrated the crucial significance of GDNF in the SSCs proliferation and self-renewal [9]. In 2003, Kanatsu-Shinohara et al. created a long-lasting in vitro culture method for mouse SSCs [10]. These results are essential for the realization of SSC transplantation in mice. However, the applicability of the results from mouse SSCs to humans is limited because of interspecies variations and other influencing variables. The identification, isolation, and in vitro culture of human SSCs are current challenges and prerequisites for achieving SSC transplantation [11]. More research is needed to understand and elucidate how human SSCs are regulated.

Various pathways responsible for the self-renewal and differentiation of SSCs were elucidated in mice. Multiple investigations have manifested that Gdnf could preserve the ability of SSCs to reproduce themselves both in vivo and in vitro [9,12] and that many changes in SSC behavior can be explained by Gdnf. Gdnf binds to the Gfra1/Ret receptor complex and activates PI3K/AKT, Ras/Erk and Src pathways to act on undifferentiated spermatogonia [13,14]. Other growth factors in the niche, such as FGFs [15], EGFs [16], and WNTs [17], are also involved in regulating SSCs. In addition, certain intracellular molecules are necessary for the self-renewal of SSCs. Plzf could enhance the SSC's ability to renew itself, and removing Plzf results in a gradual decline in the number of germ cells in the testes [18]. Plzf controls the function of the differentiation-related gene Sall4, which in turn suppresses the expression of differentiation genes either directly or indirectly [19]. Moreover, Plzf inhibits the mTORC1 pathway by upregulating Ddit4 [20]. Foxo1 [21], Taf4b [22], Nanos2 [23], and Nedd4 [24]also impact the preservation of SSCs' self-renewal. Deletion of any of these genes leads to SSC depletion.

Human SSCs differ from mouse SSCs in their type and biology, which limits the applicability of some findings from mice to humans. The spermatogenic epithelial cycle in mice is often categorized into 12 phases, whereas in humans, it is commonly classified into 6 stages [11,25]. Mouse undifferentiated spermatogonia can be classified into A_s, $A_{paired,}$ and $A_{aligned}$, and although controversial, it is generally assumed that SSCs reside in the As population [26]. Human undifferentiated spermatogonia, on the other hand, are classified into Adark and A_{pale} based on the degree of hematoxylin staining, with A_{dark} being considered as a quiescent stem cell and A_{pale} as an activated stem cell. Moreover, several molecules have been shown to have different expression patterns in humans and mice, such as OCT4 [27] and FBXW7 [28]. Direct studies on self-renewal and proliferation of human SSCs are challenging, but a few recent reports have revealed some regulators of proliferation and apoptosis in human SSC lines. Hsa-miR-1908-3p regulates the proliferation and apoptosis of human SSCs by selectively targeting KLF2 [29]. FOXP4 has a significant great level of expression in human SSCs and facilitates the process of SSC proliferation [30]. In our earlier investigation, we also discovered that TCF3 is predominantly expressed in human SSCs. It enhances SSC proliferation by increasing the transcription of PODXL [31]. However, the regulatory mechanisms of human SSC proliferation and self-renewal are still poorly understood.

In order to examine the control of human SSC proliferation and self-renewal, we conducted a comprehensive study of scRNA-seq data obtained from the testes of healthy individuals. We identified many genes that were highly expressed in SSCs, among which EEF1B2 showed the highest expression in SSCs and gradually decreased with SSC differentiation. EEF1B2 encodes a translation elongation factor that is part of the EEF1 complex and is involved in protein synthesis [32]. EEF1B2 assists in the transportation of aminoacylated tRNA to the ribosome via its interaction with eEF1A, which binds GTP and catalyzes the exchange of guanine nucleotides with eEF1A [33]. EEF1B2 mutations have been linked to neurodevelopmental disorders, but their role in male spermatogenesis is unknown [34].

In our study, we performed an integrated analysis of Single cell RNA sequencing (scRNA-seq) data from GSE112013 [35] and GSE149512 [36] and found that EEF1B2 exhibited a significant great level of expression in human SSCs, which was confirmed using immunohistochemistry. EEF1B2 knockdown in SSC lines mitigates cell proliferation and self-renewal and triggers apoptosis. RNA sequencing (RNA-seq) revealed that EEF1B2 deficiency suppressed the expression of TAF4B. Restoring TAF4B expression in SSC lines rescued the phenotypes caused by EEF1B2 deficiency. Moreover, we detected EEF1B2 downregulation in some NOA patients, suggesting that EEF1B2 may contribute to NOA progression. Our findings provide fresh perspectives on the epigenetic control of SSCs and prospective treatment targets for male infertility.

2. Materials and methods

2.1. Human testicular tissue collection

The project received clearance and oversight from the Ethics Committee of the Reproductive & Genetic Hospital at CITIC-Xiangya, which is associated with the Basic Medical Science School of Central South University in Changsha, Hunan. The authorization number for this study is LL-SC-2020-028. All participants provided informed consent. Approximately 30 mg of testicular tissue was obtained from each of the 20 azoospermic patients who underwent testicular biopsy, with ages ranging from 25 to 52 years. The tissues underwent three rounds of washing with phosphate-buffered saline (PBS) and were either treated with 4 % paraformaldehyde for fixation or rapidly frozen using liquid nitrogen.

2.2. Human testicular scRNA-seq data acquisition and analysis

Human testis single-cell RNA sequencing data were retrieved from GEO DataSets, and eight normal human testis samples from GSE112013 and GSE149512 were analyzed to investigate the development of SSCs. The Seurat, a R package) was employed to import and manipulate the expression matrix [37]. Exclusion criteria included cells with a gene expression count of less than 500 or readings mapping to mitochondrial genomes exceeding 20 %. Ribosomal genes were eliminated through a manual approach. After variable characteristics in each test were identified, the IntegrateData function was employed to merge the data sets and rectify batch effects. Uniform Manifold Approximation and Projection (UMAP) clustering and principal components (PCs) 1–12 for all genes were computed on the integrated dataset. Cell types were assigned to the various clusters according to the molecular markers' expression. To address the study objectives, the cell clusters annotated as SSCs were re-clustered, and three stages of SSCs were distinguished. The Monocle 3 software tool was employed to mimic the developmental trajectory of SSCs [38], and the genes differentially expressed at different stages were analyzed. The plot1cell [39] and dittoseq R packages [40] were used to optimize the images, and the flowcharts were created using the plotting tools from home for researcher.

2.3. Culture of human SSCs line

The human SSCs line was obtained as a donation from Professor Zuping He of Hunan Normal University, Changsha, China [41]. The cells were cultivated in DMEM/F12 media (Gibco, Grand Island, NY, USA) enriched with 10 % FBS (Gibco) and 100 units of penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 34 °C in a 5 % CO2 incubator. The cells were regularly propagated every 2–3 days using a solution of 0.05 % trypsin-EDTA (Invitrogen).

2.4. RNA extraction and qPCR

Cells were subjected to RNA isolation using RNAiso Plus reagent (Takara, Kusatsu, Japan) based on the manufacturer's protocol. The measurement of RNA quality and concentration was conducted using Nanodrop (Thermo Scientific, Waltham, MA, USA). The synthesis of cDNA from total RNA was executed employing a First Strand cDNA Synthesis Kit (Thermo Scientific) according to previously established methods by RT-PCR. The qPCR analysis was conducted on the Applied Biosystems ABI Prism 7700 equipment (Applied Biosystems, Foster City, CA, USA) using the SYBR Premix Ex *Taq*II (Takara) following the instructions provided by the manufacturer. The internal control used was ACTB. The calculation of relative mRNA expression was performed using the $\triangle\triangle$Ct technique. Every sample was tested three times. The research created primer sets for qPCR and provided a list of them in Table S1.

2.5. Protein extraction and western blot

The cells and testicular specimens were disrupted using Radio immunoprecipitation assay (RIPA) lysis solution (Thermo Scientific) while being kept on ice for 15 min. Afterward, they underwent centrifugation at a force of 12000 g for 15 min. The protein concentrations of each specimen were ascertained employing a bicinchoninic acid assay kit (Thermo Scientific). Afterward, the specimens were subjected to SDS-PAGE to separate them and then transferred onto membranes for Western blotting. The antibodies employed in this investigation are delineated in Table S2. The protein bands were detected employing chemiluminescence (Bio-Rad).

2.6. Immunohistochemistry and immunofluorescence

The testis sections underwent deparaffinization using xylene and rehydration with ethanol. They were then subjected to heating in a sodium citrate buffer (0.01 M) at 98 °C for 18 min for immunohistochemistry. The endogenous peroxidase activity was neutralized using a 3 % solution of H2O2 (Zsbio, Beijing, China), and the tissues were made permeable by treating them with a 0.25 % solution of Triton X-100 (Sigma, Steinheim, Germany) for a duration of 15 min. Following a 1-h blocking step using a 5 % BSA solution, the tissues were subjected to an overnight incubation at 4 °C using the primary antibodies specified in Table S2. The sections were washed with PBS and then incubated with HRP-conjugated secondary antibodies for 1 h at ambient temperature. The signal was produced with a DAB chromogen kit (Dako, Glostrup, Denmark). Subsequently, the slices were stained with hematoxylin. The immunofluorescence technique included incubating Alexa Fluor-conjugated secondary antibodies overnight at ambient temperature and subsequent counterstaining with DAPI. The sections were then scrutinized and evaluated using a Carl Zeiss microscope from Germany.

2.7. Cell transfection

The synthesis and design of each siRNA, as indicated in Table S3, were undertaken by Zorin, a company based in Shanghai, China. The plasmids TAF4B-flag and pCMV3-flag were acquired from SinoBiological (Beijing, China) (Supplementary Fig. 1). As directed in the manual, siRNAs (100 nmol/L) or 2.5 μg plasmid were transfected into human SSC lines using Lipofectamine 3000 transfection reagent (Life Technologies, CA, UAS). The efficiency of cell transfection was calculated by transfecting Cy3-tagged siRNA or plasmids carrying GFP. The overall transfection efficiency was approximately 80 %. Protein and RNA were extracted from cells that had been collected 48 h post-transfection in preparation for PCR and Western blot analysis.

2.8. The EdU incorporation assay

The EdU (RiboBio) reagent, at a concentration of 50 μM, was introduced into the cell culture medium and allowed to incubate for a duration of 12 h, following the instructions provided in the handbook. The human SSC lines were rinsed with DMEM and then treated with 4 % PFA for fixation. The specimens were rendered neutral using glycine (2 mg/ml) and made permeable with 0.5 % Triton X-100 for a duration of 10 min at RT. Apollo was utilized for chromatic enhancement, while cell nuclei were stained with DAPI as a counterstain. Fluorescence microscopy (Zeiss) was used to acquire and evaluate the images. A minimum of 500 cells were encompassed for statistical analysis.

2.9. Cell counting kit-8 assay

The proliferation rate of the human SSC line was assessed utilizing the CCK8 kit based on the instructions provided in the manual (Dojindo, Kumamoto, Japan). In summary, a 10 % concentration of CCK-8 reagent was introduced to the culture mix and left to incubate for a duration of 3 h. The medium's optical density was estimated at a wavelength of 450 nm employing a microplate reader (Thermo Scientific).

2.10. RNA sequencing

Personalbio, a biotechnology company in Shanghai that provides various sequencing services, performed the RNA sequencing of this project. In summary, the whole RNA's polyA structure was concentrated by utilizing Oligo(dT) magnetic beads, followed by fragmentation of the RNA into pieces around 300 base pairs in length using ion disruption. The first cDNA strand was generated by using 6-base random primers and reverse transcriptase to transcribe RNA as a template. Afterward, the second cDNA strand was produced by using the original cDNA strand as a template. Following the development of the library, PCR amplification was used to enrich the library fragments. Subsequently, library selection was carried out based on the fragment size, with the library size set at 450 bp. The library was subjected to a quality assessment utilizing the Agilent 2100 Bioanalyzer, which measured both the total concentration and the efficient concentration of the library. Libraries containing distinct index sequences, where each sample was assigned a unique index, were combined in a ratio determined by the library's effective concentration and desired data quantity. The library containing a mixture of different components was evenly thinned down to a concentration of 2 nM. Subsequently, the library was chemically treated with alkali to break down its structure and convert it into a single-stranded form. Following the extraction and purification of RNA, libraries were constructed and subjected to paired-end (PE) sequencing utilizing second-generation sequencing technology (NGS) on the Illumina sequencing platform.

2.11. Flow cytometry for apoptosis detection

Forty-eight hours after transfection, human SSC lines were harvested and then washed twice with ice-cold PBS. In accordance with the manual's instructions, Annexin V binding reagent (BD Biosciences, San Jose, CA, USA) was utilized to resuscitate at least one million cells. The cells were incubated with 10 μl of PI and 5 μl of Annexin V reagent labeled with APC for 15 min at RT while being shielded from light. The cells that had been stained were examined using a C6 flow cytometer (BD Biosciences).

2.12. Statistical analysis

The data analyses were conducted using the R programming language and the dplyr packages, which can be found at https://dplyr.tidyverse.org. The experiment was replicated a minimum of three times. The data were presented in the form of the mean ± standard deviation. The *t*-test was used to assess the disparities between the groups. A significant level of $P < 0.05$ was observed.

3. Results

3.1. Single-cell sequencing data analysis of normal adult testes

The adult testicular single-cell RNA sequencing data were obtained from GEO databases and used to explore the processes that regulate human SSC formation. The Seurat R package was employed to filter out low expression samples and samples with a high proportion of mitochondrial genes (>20 %), resulting in 22075 features across 4937 samples. After data normalization, 2000 highly

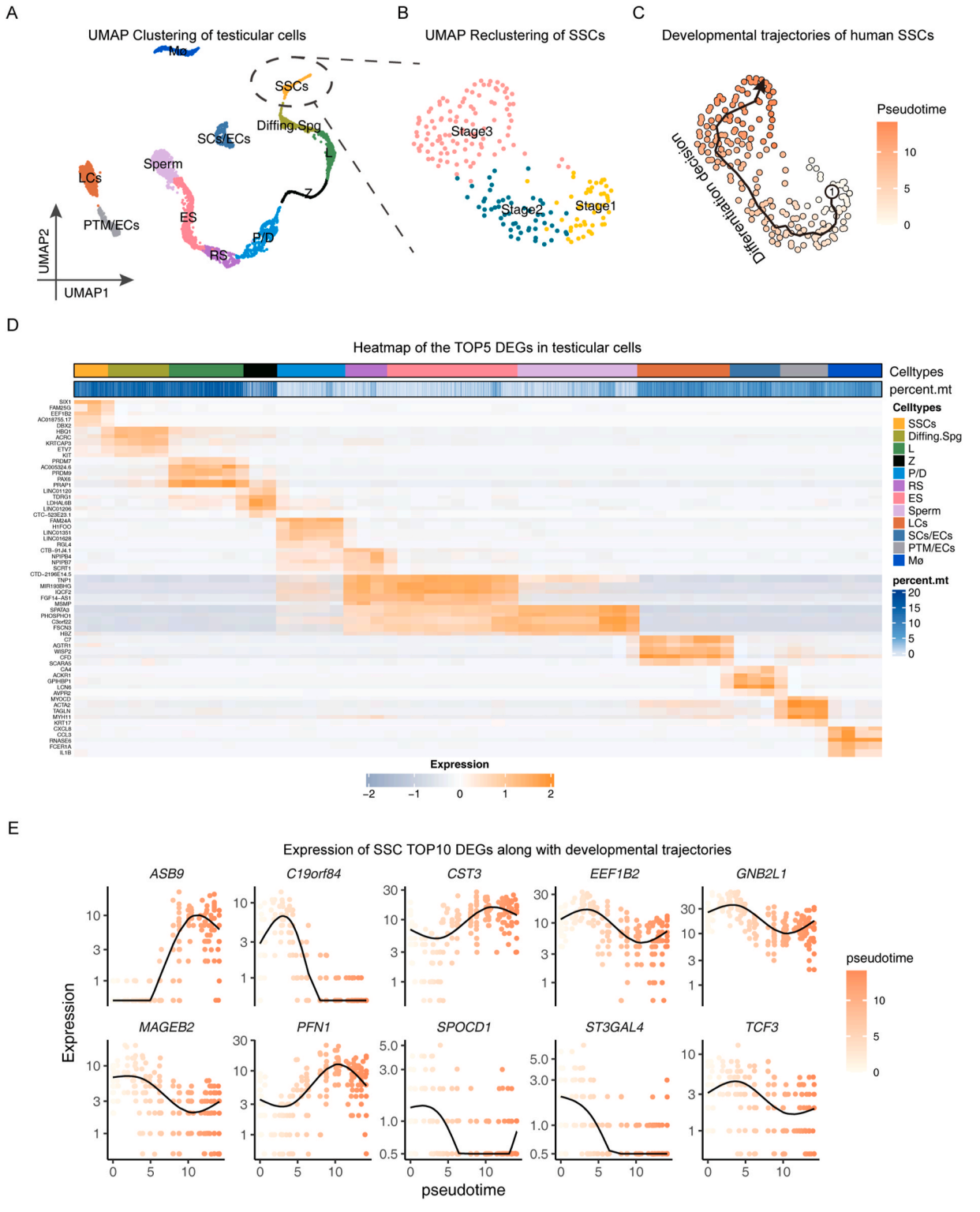

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Fig. 1. UMAP clustering of human testicular cells and analysis of differentially expressed genes in spermatogonial stem cells. (A) UMAP clustering of all testicular cells, showing 12 distinct clusters. (B) Reclustering of SSCs, classifying them into three developmental stages (stages 1–3). Developmental trajectory of SSCs, with character 1 indicating the initial state and cells differentiating continuously along the arrow direction. The darker orange color denotes higher differentiation. (D) Heatmap of differentially expressed genes in all testicular cells. The orange color indicates the expression level. (E) Projections of SSC differentially expressed genes on developmental trajectories. Each dot represents a cell. The orange color reflects the differentiation level. SSCs: spermatogonial stem cells; Diffing. spg: differentiating spermatogonia; L: leptotene spermatocytes; Z: zygotene spermatocytes; P/D: pachytene/diplotene spermatocytes; RS: round spermatids; ES: elongated spermatids; LCs: Leydig cells; SCs/ECs: Sertoli cells/epithelial cells; PTM/ECs: peritubular myoid cells/epithelial cells; Mø: macrophages.

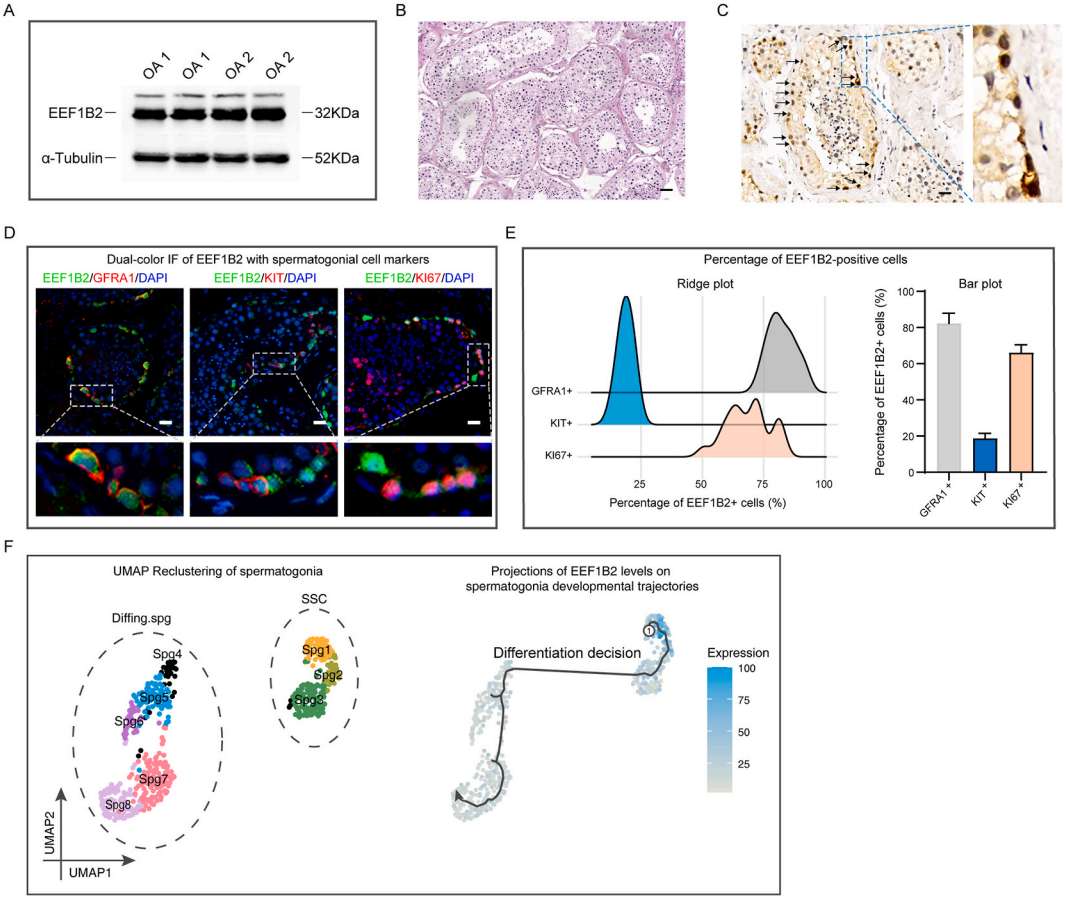

Fig. 2. EEF1B2 expression in normal testis. (A) Western blot experiments revealed that EEF1B2 is expressed in testes with normal spermatogenesis. Two OA testis samples (normal spermatogenesis), one repetition for each sample. (B) HE staining of testicular sections of OA samples showing normal spermatogenesis. Scale bar, 50 μm. (C) Distribution pattern of EEF1B2 in the testis detected by immunohistochemistry. Scale bar, 50 μm. (D) Immunofluorescence images of EEF1B2 and different types of spermatogonia markers. Including SSC marker GFRA1, differentiated spermatogonial cell marker KIT, and proliferating cell marker KI67. Green is EEF1B2; red is the respective marker molecules. Scale bar, 20 μm. (E) Ridge and bar graphs demonstrating the proportion of EEF1B2 co-expressed with the three markers in D. (F) Spermatogonial subpopulation re-clustering and eef1b2 expression trajectory. Left: Re-clustering of spermatogonia. All spermatogonia were clustered into 8 populations, including 3 SSC clusters and 5 differentiated spermatogonia clusters. Right: Expression of EEF1B2 along the developmental trajectory of spermatogonia. The solid black line represents the trajectory of cell development, and the arrow is the direction of differentiation. Each dot represents a cell. The color blue (or gray) indicates a high (or minor) degree of expression, as seen on the color legend at the right.

variable genes were selected for dimensionality reduction, followed by 50 PCs for UMAP clustering, which classified all testicular cells into 12 classes (Fig. 1A). Based on our previous studies, ID4, PIWIL4, and NANOS3 were chosen to identify SSCs [42], DMRT1, STRA8 and KIT to identify differentiated spermatogonia, SYCP1, SPO11 and OVOL2 to identify spermatocytes at different stages, TNP2 and PRM2 to identify spermatids of different types, AMH and WT1 to find Sertoli cells, DCN and MYH11 to find peritubular myoid cells, INSL3 to identify Leydig cells, VWF to identify epithelial cells and CD68 and CD14 to identify macrophages. The data of SSCs were extracted and re-clustered and three different stages of SSCs were distinguished (Fig. 1B). The developmental trajectories of SSCs were analyzed using the Moncle3 R package and the developmental starting point of SSCs was identified based on the levels of PIWL4 and NANOS3 (Fig. 1C). The differentially expressed genes (DEGs) in 12 clusters of testicular cells and three types of SSCs were further

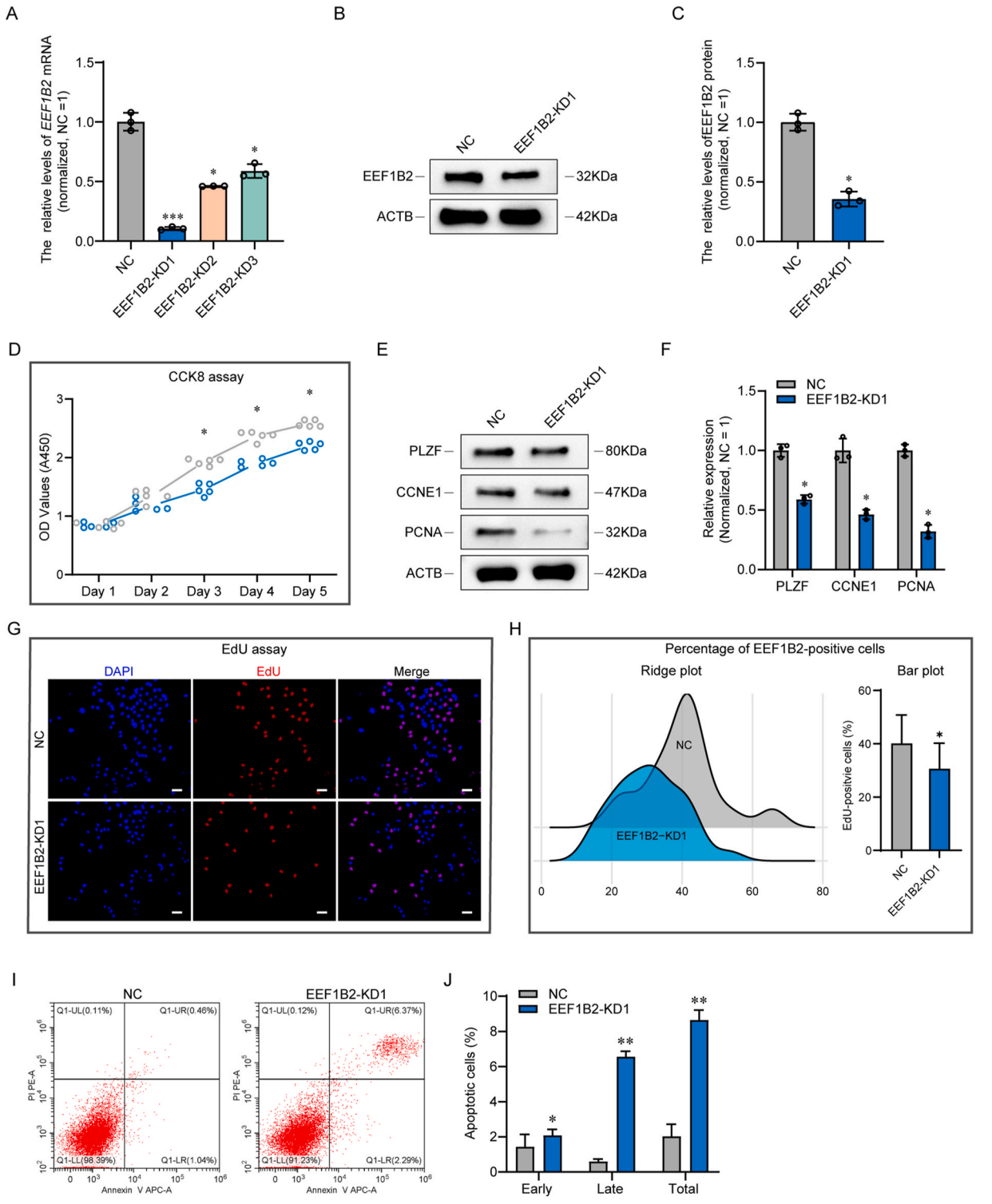

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Fig. 3. The function of EEF1B2 in the proliferation and apoptosis of human SSC lines. (A) qPCR demonstrates EEF1B2 mRNA levels after the knockdown of three siRNAs. (B) Western blot detection of protein levels of EEF1B2 after siRNA knockdown. (C) Bar graph showing relative protein levels of EEF1B2 in B. (D) CCK8 assay detects cell proliferation from 1 to 5 days after EEF1B2 knockdown. (E) Western blot assay to quantify the expression of SSC self-renewal and proliferation-related proteins following EEF1B2 knockdown. Including PLZF, CCNE1, and PCNA. (F) Bar graphs demonstrate the PLZF, CCNE1, and PCNA relative levels in E. (G) Fluorescence images of EdU incorporation. Red is the positive EdU signal. Nuclei were counterstained with DAPI. (H) Ridge and bar graphs showing the proportion of EdU-positive cells in G. (I) Flow cytometry was performed to detect Annexin V-positive apoptotic cells. (J) Bar graph showing the proportion of apoptotic cells of each type. * indicates $P < 0.05$. ** represents $P < 0.01$.

analyzed. The heatmap showed the expression of top 5 DEGs in 12 clusters of cells (Fig. 1D), and the dot plots showed the trend of Top10 genes in SSCs along their developmental trajectories (Fig. 1E). The roles of TCF3 [31], ASB9 [42] and MAGEB2 [43] in human SSC were reported in previous studies. Here, EEF1B2 manifested significant upregulation in SSCs, and its overall level was gradually decreased along the process of differentiation decision. These data suggest that EEF1B2 may be involved in the early development of SSCs, encompassing proliferation and self-renewal.

3.2. EEF1B2 distribution in human testicular tissues

To confirm the outcomes of the bioinformatics study, we investigated the dispersion of EEF1B2 in healthy human testicular tissues. In the testes of obstructive azoospermia patients, we found that EEF1B2 had a high overall expression level by Western blot experiments (Fig. 2A). In testes with normal spermatogenesis, immunohistochemistry showed that Cells positive for EEF1B2 were mostly located in close proximity to the basement membrane of the seminiferous tubules (Fig. 2B and C). Next, we performed a two-color immunofluorescence assay using multiple spermatogonia markers with EEF1B2, and consistent with the bioinformatics analysis, EEF1B2 was predominantly expressed with SSC (GFRA1-positive) and was significantly reduced in differentiated spermatogonia (KIT-positive). We also found that EEF1B2 co-localized with the cell proliferation marker KI67, emerging that EEF1B2 may be involved in the proliferation of SSC (Fig. 2D and E). In addition, we also extracted single-cell sequencing data of all spermatogonia and clustered them again to obtain 3 clusters of SSC and 5 clusters of differentiated spermatogonia. Developmental trajectories were constructed using monocle 3, and heat maps of EEF1B2 on the developmental trajectories were drawn (Fig. 2F). Consistent with the outcomes of immunofluorescence, the EEF1B2 expression was mostly seen in SSC and gradually down-regulated in differentiated spermatogonia.

3.3. The function of EEF1B2 in the proliferation and apoptosis of human SSC lines

The human SSC line, a gift from He's lab, which expresses many human SSC markers and has a similar biology to primary SSCs [41], was employed to ascertain the impact of EEF1B2 on the proliferation and apoptosis of SSCs. EEF1B2 was knocked down in the SSC line by transfecting siRNA, and the expression level was verified by qPCR (Fig. 3A) and Western blot (Fig. 3B and C). Cell proliferation was examined using CCK8 for five consecutive days, and a significant suppression of cell proliferation was found starting on the second day after transfection, lasting until the fifth day (Fig. 3D). Multiple proteins linked to the proliferation and self-renewal of SSCs include as PLZF, CCNE1, and PCNA, were detected 48 h after transfection, and their levels were significantly downregulated (Fig. 3E and F). The effect of EEF1B2 downregulation on cellular DNA synthesis was examined by EdU incorporation assay, and a significant inhibition of DNA synthesis was found (Fig. 3G and H). Apoptosis of cells was also examined due to a large amount of debris in cell culture following EEF1B2 knockdown, and a significant upregulation of apoptosis was induced by EEF1B2 insufficiency (Fig. 3I and J). These data suggest that EEF1B2 promotes the proliferation of SSC lines in vitro, which is consistent with the previous hypothesis.

3.3.1. RNA sequencing screening of downstream genes of EEF1B2

To ascertain the contribution of EEF1B2 in regulating SSC proliferation and apoptosis, we knocked down EEF1B2 expression in SSCs and performed RNA sequencing. After filtering out low expression and blank values, 14,802 genes were selected for further study. Out of the total, 21 genes exhibited elevation, while 23 genes were hindered ($p < 0.05$, fold changes >2). The heatmap of the top 50 DEGs demonstrated the consistency of the sequencing data within and between groups (Fig. 4A). The volcano plot showed the expression levels of all genes, with 21 elevated genes in red and 23 mitigated genes in blue (Fig. 4B). We conducted KEGG (Fig. 4C) and GO (Fig. S1) enrichment analysis on the down-regulated genes and Mfuzz trend analysis (Fig. S2) on all genes. The results indicated that the down-regulated genes were mainly enriched in signaling pathways encompassing Tight junction, and the genes with a down-regulation trend were mainly associated with biological processes such as cell junction assembly and ribosome biogenesis. We confirmed the trustworthiness of RNA sequencing by validating the differential expression of certain genes employing qPCR and seeing a strong agreement with the RNA sequencing findings (Fig. 4D). Moreover, we integrated data from scRNA and Bulk-RNA sequencing to examine the localization of all significantly down-regulated genes in the testis. We observed that C11orf65, TAF4B, TJP2, LOM2, and UHMK1 were highly expressed in the SSCs, especially TAF4B and TJP2 (Fig. 4E). We further measured the TAF4B and TJP2 expression levels via Western blot and detected a more pronounced downregulation of TAF4B after EEF1B2 knockdown (Fig. 4F). These findings suggested that TAF4B might be a downstream functional gene of EEF1B2.

3.4. TAF4B re-expression alleviates phenotypic defects caused by EEF1B2 knockdown

To explore the involvement of TAF4B in EEF1B2-mediated SSC proliferation regulation, we knocked down EEF1B2 expression and

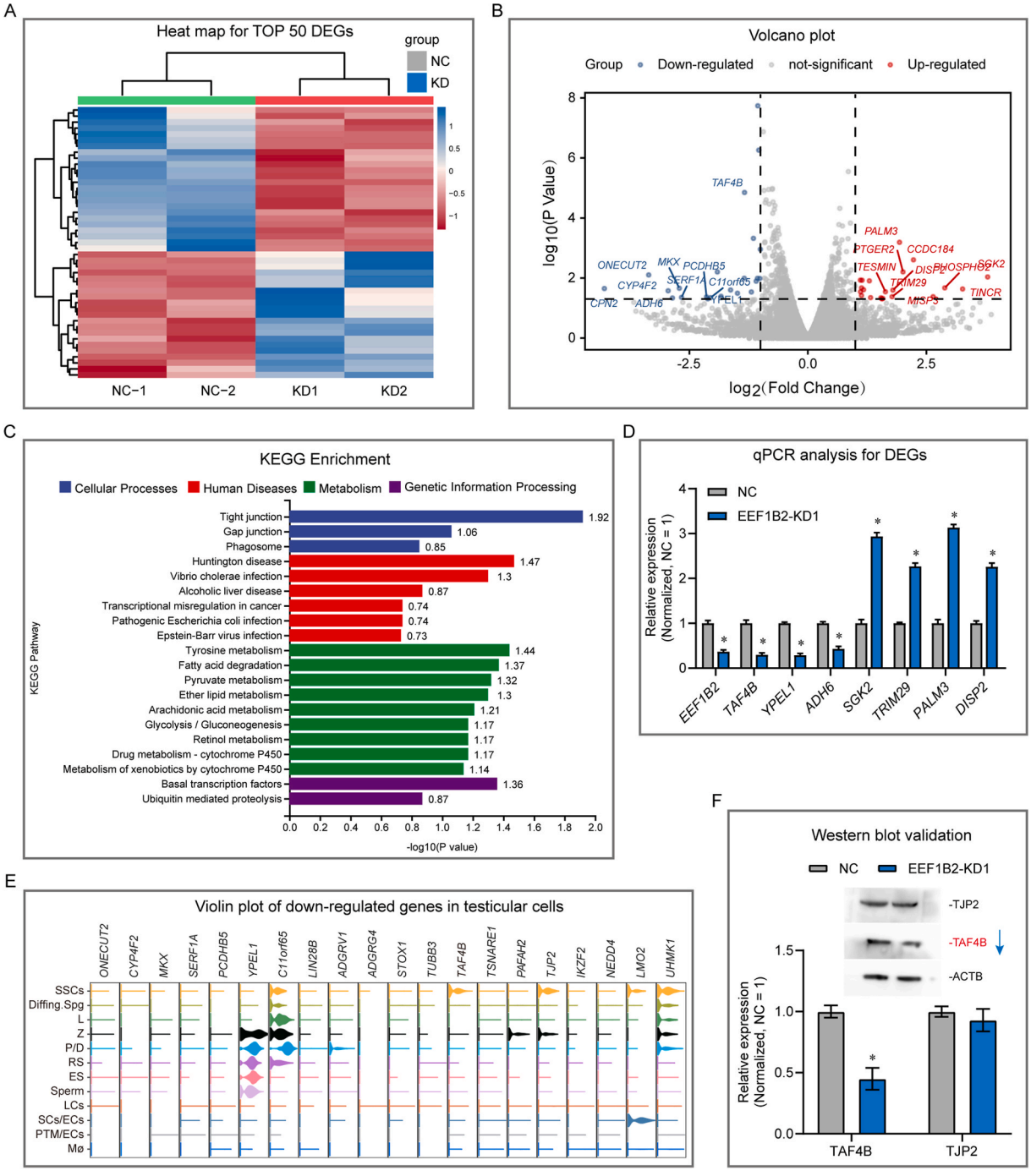

Fig. 4. RNA sequencing to detect signaling pathways and downstream genes involved in EEF1B2. (A) Heatmap showing the distribution of the Top 50 DEGs in each sample. The Z score at the right indicates the color of the scaled gene expression levels. (B) Volcano plot depicting the expression distribution of all genes. Top 20 DEGs were annotated. Red indicates elevated genes, blue manifests the mitigated genes, and gray denotes genes that have not undergone significant change. (C) KEGG terms enriched in all DEGs. (D) qPCR was executed to validate the levels of randomly selected genes. (E) Distribution of Top 20 DEGs in the single-cell atlas of testis in Fig. 1A. (F) Western blot was conducted to quantify the TJP2 and TAF4B levels. * indicates $P < 0.05$.

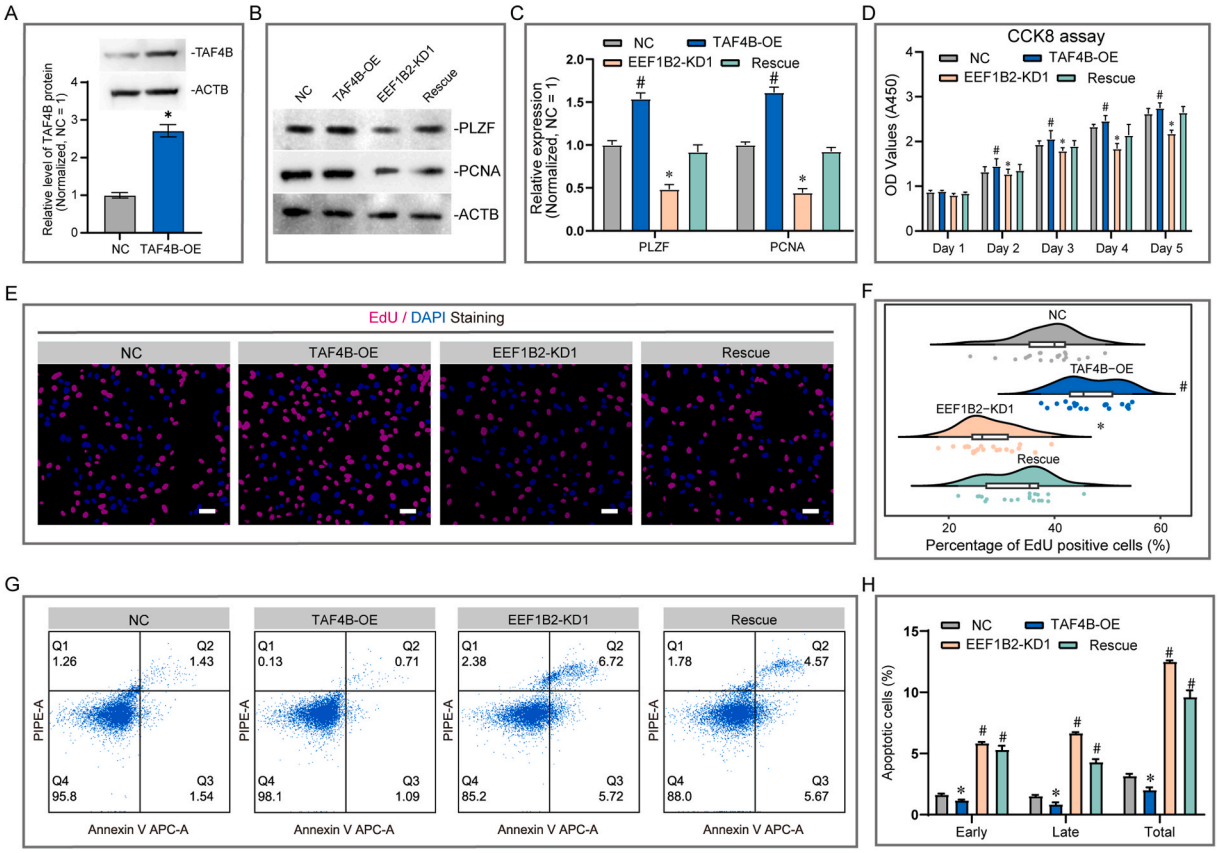

Fig. 5. TAF4B overexpression alleviates phenotypic defects caused by EEF1B2. (A) Western blot detection of TAF4B protein levels after overexpression. (B) Western blot quantified the PLZF and PCNA expression levels in 4 groups. The control group (NC), TAF4B overexpression group (TAF4B-OE), EEF1B2 knockdown group (EEF1B2-KD1), and EEF1B2 knockdown and TAF4B overexpression combined group (Rescue) were included. (C) Bar graphs showing the relative levels of PLZF and PCNA in each group. (D) CCK8 assay for cell proliferation in each group at 1–5 days. (E) EdU incorporation detects DNA synthesis in each group. (F) Cloud rain plot showing the proportion of EdU-positive cells in each group in E. (G) Flow cytometry to detect apoptotic cells in each group. (H) Bar graph showing the percentage of different types of apoptotic cells in each group. # represents a significant elevation compared to the control group. * represents a significant hindrance compared to the control.

restored it by transfecting EEF1B2 plasmid. We assessed the phenotypic changes of the cells by Western blot and found that TAF4B expression was significantly increased by TAF4B re-expression (Fig. 5A). We divided the cells into four groups: negative control (NC), TAF4B overexpression, EEF1B2 knockdown, and rescue group (EEF1B2 knockdown + TAF4B overexpression). We quantified the PLZF and PCNA protein's expression levels and manifested that TAF4B overexpression enhanced their expression, indicating increased cell proliferation and self-renewal. In addition, TAF4B overexpression reversed the down-regulation of PLZF and PCNA caused by EEF1B2 knockdown (Fig. 5B and C). CCK8 (Fig. 5D) and EdU assays (Fig. 5E and F) confirmed that TAF4B overexpression stimulated proliferation in SSC lines and rescued the proliferation impairment induced by EEF1B2 knockdown. Furthermore, flow-combined Annexin V staining revealed that TAF4B overexpression reduced apoptosis triggered by EEF1B2 knockdown (Fig. 5G and H). These results suggested that TAF4B re-expression mitigated the phenotypic defects resulting from EEF1B2 knockdown, implying that TAF4B is a downstream functional gene of EEF1B2.

3.5. *EEF1B2 is downregulated in the testes of azoospermia patients*

Prior investigations have shown that deficiency of many genes, such as Plzf [18], Foxo [21], Dot1l [44], and Carf [45], impaired spermatogenesis by regulating SSC. Whether EEF1B2 is associated with human azoospermia is unknown. To investigate this, we examined the EEF1B2 expression in various types of NOA testes, such as hypospermatogenesis (HS), spermatid maturation arrest (Std MA), and spermatocyte maturation arrest (Spc MA). Two-color immunofluorescence revealed that EEF1B2 expression was markedly decreased in NOA testes, particularly in those with severe spermatogenesis impairment, such as Spc MA (Fig. 6A and B). We also analyzed testicular single-cell sequencing data from NOA patients (GSE149512) and manifested that NOA specimens had a significantly lower EEF1B2 expression than in normal testis (GSE112013) (Fig. 6C). These outcomes emerged that EEF1B2 deficiency might be linked to azoospermia.

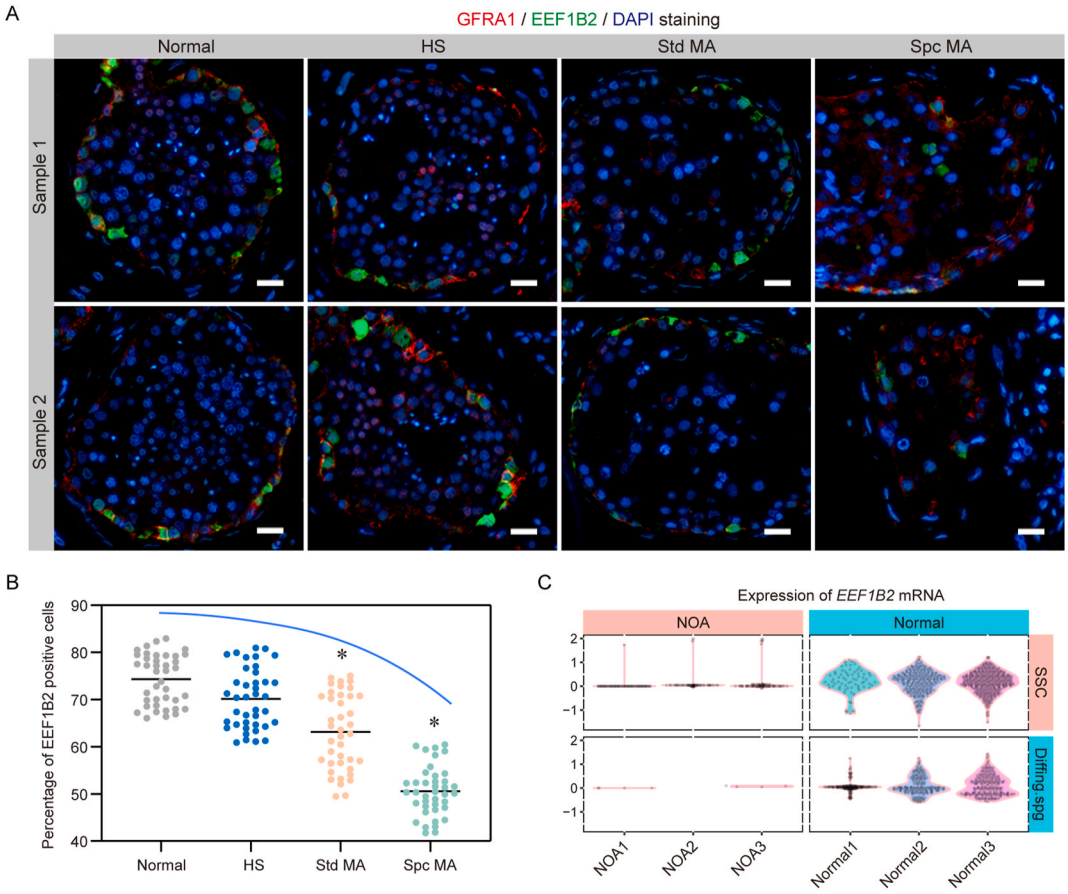

Fig. 6. EEF1B2 expression in patients with NOA. (A) Immunofluorescence images of EEF1B2 and GFRA1 in patients with different types of NOA. Including normal, hypospermatogenesis, Spermatid maturation arrest, and spermatocyte maturation arrest. Red represents GFRA1, and green represents EEF1B2. Scale bar, 20 μm. (B) Dot plot illustrating the percentage of EEFB12-positive cells in the SSC of each sample in A. (C) Violin plots illustrating the expression levels of EEFB12 in single-cell transcriptional profiles of SSCs and differentiated spermatogonia from NOA and normal testis. * indicates $P < 0.05$.

4. Discussion

SSCs are responsible for the initiation of spermatogenesis and may be used to regenerate the germline lineage inside the testes of mice [8]. Nevertheless, the use of human SSCs in reproductive regenerative medicine encounters several obstacles. It is not possible to cultivate human SSCs in vitro for the long term, and the optimal culture conditions for their proliferation and self-renewal are unknown [46]. The isolation and long-term culture of human SSCs are pressing issues that need to be addressed. Previous studies have revealed several key factors that sustain the proliferation and self-renewal of SSCs, such as GDNF, FGF, and LIF [47]. These conditions facilitate the in vitro cultivation of mouse SSCs, but they are insufficient for human SSCs, which may require additional signals to maintain their homeostasis. To identify the regulative signals of human SSCs, we examined published single-cell sequencing data of human testis. We found that the SSCs had a great expression of protein EEF1B2 and decreased progressively during the development of spermatocytes and SSCs, indicating that it might have a regulatory role in the early stage of SSCs.

EEF1B2 is a eukaryotic translation elongation factor that forms a complex with three other proteins (EEF1A1, EEF1A2, and EEF1D) and participates in the elongation process of translation [32]. EEF1B2 has been mainly studied in the fields of cancer and neuroscience [48], and several investigations have manifested that EEF1B2 is essential for neural development, and its biallelic mutations cause intellectual disability [49]. EEF1B2 is also upregulated in most cancers and may be associated with poor prognosis, but some studies have reported contradictory results, such as low expression of EEF1B2 in gliomas correlating with low survival rate and low levels of EEF1B2 in hepatocellular carcinoma being associated with high survival rate [32]. Moreover, EEF1B2 is considered as a potential cellular senescence marker, as it is significantly downregulated in ionizing radiation-induced senescent cells [50]. Despite these studies suggesting the possible functions of EEF1B2 in tumorigenesis and normal development, some data are still inconclusive and require more refined cellular and molecular experiments to verify its function. In this paper, we observed that EEF1B2 was highly expressed in SSCs at early developmental stages and decreased gradually during differentiation. Cell proliferation was inhibited, and apoptosis was enhanced in human SSC lines upon EEF1B2 knockdown. These results were consistent with previous data in tumor cells, but the role of

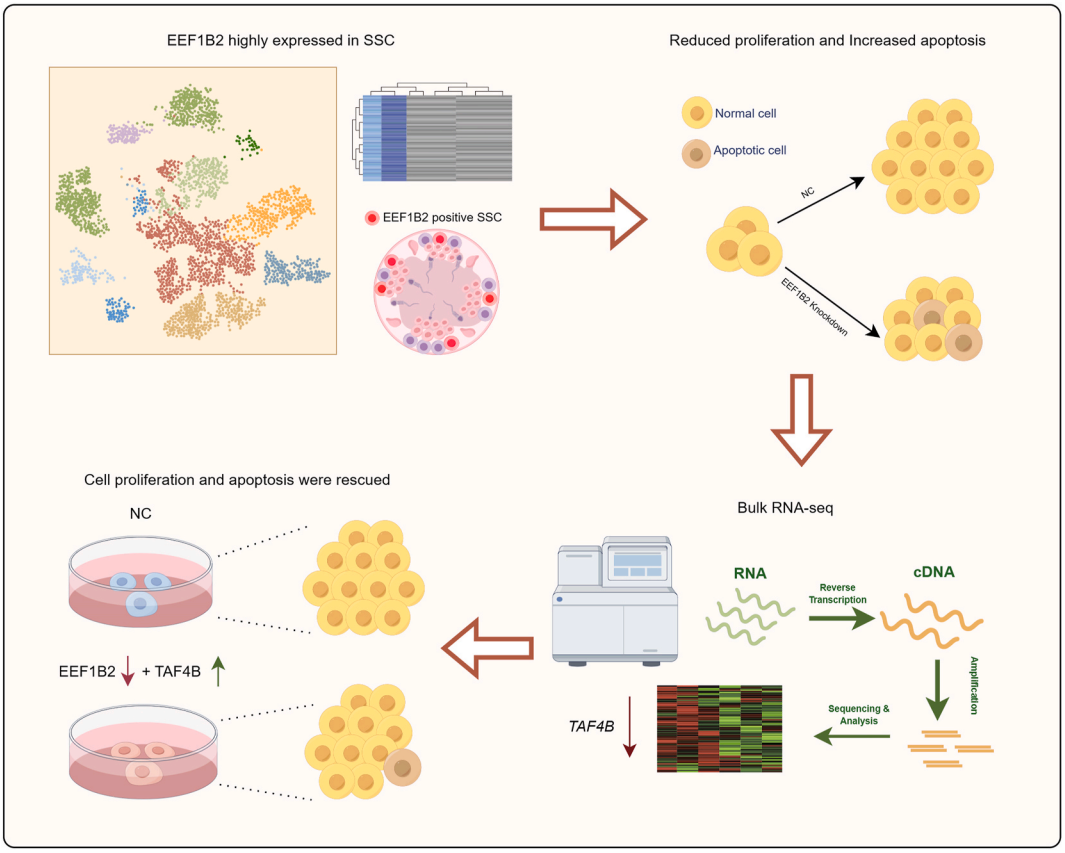

Fig. 7. Schematic diagram of the experimental workflow of this study.

EEF1B2 in physiological conditions remains elusive due to ethical issues. We will use mouse SSC transplantation experiments to investigate the effects of EEF1B2 on SSC self-renewal and differentiation under physiological conditions. As a protein translation elongation factor, EEF1B2 mainly regulates protein translation, and we aim to uncover its target genes and mechanisms by RNA binding protein immunoprecipitation and RNA sequencing. Due to the poor specificity of the antibody, we did not enrich significantly different mRNA. In this study, we only identified some genes that were indirectly regulated by EEF1B2 by RNA sequencing. The genes that are directly involved in translation by EEF1B2 are still unknown, and this is an important question that we will attempt to answer in the future.

In our RNA sequencing results, we validated the expression of two DEGs that were highly expressed in SSCs, TAF4B, and TJP2, and showed that TAF4B is a functional downstream protein of EEF1B2. C11orf65, LMO2, and UHMK1 were also abundant in other testicular cells, but their role in EEF1B2 regulation of SSCs remains unclear. TAF4B is a transcription factor that is enriched in gonads and required for mouse SSC self-renewal. TAF4b-deficient spermatogenic progenitor cells differentiate prematurely and fail to establish an SSC pool during the critical developmental window [51]. We demonstrated that TAF4B is also important for human SSCs, but its conserved function in human and mouse SSCs needs further investigation.

In mice, functional defects in SSCs usually result in the gradual depletion of spermatogenic cells and lead to azoospermia. To explore whether EEF1B2 is associated with human azoospermia, we analyzed scRNA data from azoospermic testes and examined the distribution of EEF1B2 in testes with different types of spermatogenic impairment. We found that EEF1B2 was downregulated in human azoospermia, suggesting that its low level may be related to azoospermia. However, these data do not prove a causal relationship between EEF1B2 and azoospermia. We also attempted to identify pathogenic mutations of EEF1B2 from whole-exome sequencing data of azoospermic patients, but no positive results were found. In addition, to elucidate the role of EEF1B2 in spermatogenesis, it is essential to generate conditional knockout mice of EEF1B2 in testes, which is one of our future research focuses.

4.1. Limitations of the study

In this study, we elucidated the molecular mechanism through which EEF1B2 modulates the proliferation and apoptosis of human SSC lines at both tissue and cellular levels via single-cell sequencing analysis and cellular experiments. Nevertheless, it is imperative to substantiate its function in vivo utilizing animal models. Although we observed a correlation between the diminution of EEF1B2 and NOA, a more extensive sample size is requisite to thoroughly investigate its role in the etiology and progression of NOA.

5. Conclusion

In summary, we report that the translation elongation factor EEF1B2 is greatly expressed in human SSCs and declines during differentiation. EEF1B2 knockdown in SSC lines reduces cell proliferation and self-renewal and increases apoptosis. By RNA sequencing, we show that EEF1B2 knockdown affects the expression of TAF4B, a transcription factor required for mouse SSC self-renewal, and we confirm their functions by rescue experiments (Fig. 7). Furthermore, we find that EEF1B2 is downregulated in azoospermic testes, indicating a possible association with azoospermia. Our data reveal new aspects of SSC development and suggest potential strategies for the diagnosis and treatment of male infertility.

Data availability statement

The data produced by this work may be acquired from the corresponding author following a reasonable inquiry.

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CRediT authorship contribution statement

Zenghui Huang: Writing – review & editing, Writing – original draft. **Ning Li:** Conceptualization. **Xiren Ji:** Investigation. **Dai Zhou:** Investigation, Funding acquisition. **Ruijun Wang:** Formal analysis. **Xingguo Zhao:** Investigation. **Huan Zhang:** Data curation. **Chuan Huang:** Writing – review & editing, Investigation. **Ge Lin:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36467.

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