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ORIGINAL ARTICLE

WNT5A regulates the proliferation, apoptosis and stemness of human stem Leydig cells via the β-catenin signaling pathway

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Abstract

Stem Leydig cells (SLCs) are essential for maintaining normal spermatogenesis as the significant component of testis microenvironment and gonadal aging. Although progress has been achieved in the regulation of male germ cells in mammals and humans, it remains unknown about the genes and signaling pathways of human SLCs. Here we have demonstrated, for the first time, that WNT5A (Wnt family member 5a) mediates the proliferation, apoptosis, and stemness of human SLCs, namely $NGFR^+$ Leydig cells. We revealed that $NGFR^+$ Leydig cells expressed NGFR, PDGFRA, NES, NR2F2, and THY1, hallmarks for SLCs. RNA-sequencing showed that WNT5A was expressed at a higher level in human SLCs than non-SLCs, while immunohistochemistry and Western blots further illustrated that WNT5A was predominantly expressed in human SLCs. Notably, CCK-8, EdU and Western blots displayed that WNT5A enhanced the proliferation and DNA synthesis and retained stemness of human SLCs, whereas flow cytometry and TUNEL analyses demonstrated that WNT5A inhibited the apoptosis of these cells. WNT5A knockdown caused an increase in LC lineage differentiation of human SLCs and reversed the effect of WNT5A overexpression on fate decisions of human SLCs. In addition, WNT5A silencing resulted in the decreases in nuclear translocation of β-catenin and expression levels of *c-Myc*, *CD44*, and *Cyclin D1*. Collectively, these results implicate that WNT5A regulates the proliferation, apoptosis and stemness of human SLCs through the activation of the β-catenin signaling pathway. This study thus provides a novel molecular mechanism underlying the fate determinations of human SLCs, and it offers a new insight into the niche regulation of human testis.

Keywords Stem Leydig cells · Human · WNT5A · β-catenin · Proliferation · Apoptosis · Stemness

Introduction

Spermatogenesis is a complex process of male germ cell development that occurs in the testis [1]. Testicular somatic cells and seminiferous tubule epithelium constitute the microenvironment or niche for the testis [2]. Leydig cells (LCs) reside in the interstitium and they secret 95% of androgen that is essential for sex differentiation, growth and development as well as spermatogenesis and muscle health [3]. Reduction of androgen synthesis and secretion by LCs in aging testicular tissues leads to hypogonadism [4], and one possible solution to this issue is exogenous testosterone replacement therapy [5]. However, it has been accompanied by an increased risk of adverse effect, e.g., polycythemia, sleep apnea, and infertility [6–8]. The immune-privileged properties of testicular tissues make it feasible for cell therapy, but mature LCs almost lose their ability of division [9]. LCs are derived from undifferentiated stem Leydig cells (SLCs), which have the capacity to produce new LCs through self-renewal and differentiation [10], as evidenced by the findings that transplanted SLCs can regenerate the senescent and chemically destroyed LCs to restore testosterone production and spermatogenesis [11]. Therefore, it is of particular significance to identify human SLCs and uncover

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molecular mechanisms that regulate the fate determinations of human SLCs.

Due to the difficulty in acquiring human testicular tissues, our understanding of SLCs largely comes from rodents whose development patterns are distinct from humans. While rodent SLCs have a biphasic pattern of development (fetal and pubertal to adult), human SLCs have a triphasic pattern including fetal, neonatal, and adult stages [12]. Over the past decade, SLCs from rodents have been effectively isolated, expanded, and differentiated in vitro [13], and several markers, including NESTIN, platelet-derived growth factor receptor alpha (PDGFRA, also known as CD140A), nuclear receptor subfamily 2 F group member 2 (NR2F2, also known as COUP-TFII), integrin subunit αV (ITGAV, also known as CD51), p75 neurotrophin receptor (NGFR, also known as CD271), and THY1 (also known as CD90) [11, 14–17], have been identified. It remains to be determined whether rodent SLC markers are suitable for identifying and isolating human SLCs from adult testes. It has been reported that $NGFR^+$ SLCs form spheres in suspension culture in vitro and differentiate into functional LCs in adherent culture since they express LC lineage markers and produce testosterone in vitro and in vivo [18]. Endosialin (also known as CD248) has been suggested to be a specific marker for human SLCs [19], while primary human testicular $PDGFRA^+$ cells can be induced to differentiate into steroidogenic cells with LCs characteristics in vitro [20].

Over the last few years, some progress has been made in uncovering molecular mechanisms underlying SLCs' differentiation and the fate decisions of human spermatogonial stem cells (SSCs). Nestin deletion leads to a decrease in the differentiation of SLCs in mice, whereas pharmacological intervention with melatonin reverses the reduction of SLCs' differentiation and alleviates the aging of the male reproductive system [21]. Leptin binds to leptin receptors of SLCs and induces desert hedgehog (DHH) signaling to regulate human SLCs' differentiation [22]. Recently, we have demonstrated that OIP5 interacts with NCK2 to mediate the self-renewal and apoptosis of human SSCs [23]. Notably, specific genes and signaling pathways that regulate the fate determinations of human SLCs remain to be elucidated.

WNT5A is a secreted glycoprotein that belongs to the Wnt protein family, and it plays a critical role in cell fate determinations, including proliferation and migration in embryonic development and adult tissues [24, 25]. The Wnt signaling mainly includes three pathways, namely the classical Wnt/β-catenin pathway, planar cell polarity pathway (PCP), and Wnt-Ca^{2+} signaling pathway [26, 27], and the function of WNT5A in various kinds of cells depends on different signaling pathways. WNT5A has been reported to be essential for a variety of physiological processes, e.g., neurodevelopment, bone formation, and immune regulation [28–30]. WNT5A plays an important role in the maintenance and differentiation of stem cells, including embryonic stem cells, SSCs, mesenchymal stem cells, neural stem cells, and hematopoietic stem cells [31]. Abnormal WNT5A expression and function are associated with degenerative diseases and tumors, e.g., Robinow syndrome, glioblastoma, breast cancer, and gastric cancer [32, 33]. Notably, WNT5A has been shown to be an important regulator for spermatogenesis. First of all, WNT5 positively regulates SSC self-renewal [34] through the β-catenin independent mechanism [35], and WNT5A derived from LCs and Sertoli cells stimulates the self-renewal of mouse type A spermatogonia [36]. Secondly, WNT5A controls the Sertoli cell junction through the PCP signaling pathway [37]. LCs can synthesize and secrete WNT5A. Nevertheless, the function and molecular mechanism of WNT5A in mediating human SLCs remain unknown.

In the current study, we analyzed human testis single-cell transcriptome data from a public database and found that the highest positive rate of NGFR was observed in human putative SLCs. $NGFR^+$ cells expressed markers for putative human SLCs. RNA-sequencing showed that the transcript of WNT5A was significantly higher in human SLCs than non-SLCs, reflecting that WNT5A is involved in fate determinations of human SLCs. We have reported for the first time that WNT5A mediates the proliferation, DNA synthesis, apoptosis, and stemness of human SLCs by activating the β-catenin pathway. As such, this study provides a novel gene and signaling pathway mediating fate determinations of human SLCs, which underlies molecular mechanisms of the testicular microenvironment.

Methods and materials

Acquirement of human testis tissues

Human testicular tissues were obtained from nine obstructive azoospermia (OA) patients with normal spermatogenesis and undergoing microdissection testicular sperm extraction (m-TESE) or castration therapy for prostate cancer at the Third Xiangya Hospital of Central South University or Hunan Cancer Hospital between September 2021 and March 2023. Tissues were washed three times with phosphate-buffered saline (PBS) containing 4% streptomycin and penicillin, and they were fixed with 4% paraformaldehyde (PFA) for immunohistochemistry or stored in liquid nitrogen for protein or RNA extraction.

Stem Leydig cells' (SLCs') isolation and culture

Testicular tissues were used to isolate SLCs and non-SLCs from testicular tissues of five OA patients (Table S1). Testicular tissues were cut into small pieces of approximately

1 × 1 × 1 mm with ophthalmic surgical scissors and digested with 2 mg/mL collagenase IV (Gibco, USA) and 500 μg/mL DNase I (Roche, USA) in DMEM/F12 (Gibco, USA) for 30 min at 34 °C in a shaking water bath (100 beats/min). The cells were filtered through a 40 μm filter after 10 min rest and centrifuged at 256 g for 4 min at 4 °C. Cell pellets were washed twice with Ca^{2+}/Mg^{2+} -free PBS, and cell mixture was seeded in 10 cm diameter cell culture dishes supplemented with 0.1% gelatin at 34 °C for 0.5–2 h in DMEM/F12 supplemented with 10% FBS (Gibco) to obtain Leydig cells (LCs). LCs were incubated with NGFR antibody (BD Biosciences, USA) and isotype IgG antibody for 60 min in the dark on a shaker. After being washed twice with PBS, the $NGFR^+$ LCs, namely SLCs, were obtained through flow cytometry (BD Biosciences), whereas the $NGFR^-$ LCs by flow cytometry sorting were non-SLCs. SLCs were cultured in the conditioned medium with DMEM/F12, 5% chicken embryo extract (Us Biologicals, USA), 1% GlutaMAX (Gibco), 1% non-essential amino acids (Gibco), 1% N2 (Gibco), 2% B27 (Gibco), 1 × insulin-transferrin-selenium (ITS) (Gibco), 20 ng/mL oncostatin M (PeproTech), 20 ng/mL PDGFBB (PeproTech, USA), 20 ng/mL epidermal growth factor (EGF, PeproTech), 20 ng/mL basic fibroblast growth factor (FGF, PeproTech), 1 nM dexamethasone (Sigma, USA), 1 ng/mL leukemia inhibitory factor (LIF, Millipore, USA), and 0.1 mM mercaptoethanol (Gibco). The SLCs were cultured under the conditions of 34 °C and a 5% CO_2 incubator, and the medium was changed every 3 days. Detailed information on antibodies was shown in Table S2.

Transfection of siRNAs and vectors

The transfection of siRNAs of WNT5A or vectors was performed with Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's protocol. Three siRNAs targeting different sequences of WNT5A were designed and synthesized from Genepharma (Suzhou, China). SLCs were collected at 48 h after siRNA transfection for detecting gene expression, while they were harvested at 72 h after siRNA transfection for protein analysis. The siRNA sequences were presented in Table S3.

Quantitative real-time PCR (qPCR) and RT-PCR

TRIzol reagent (Takara, Japan) was used to lyse cells, and total RNA was extracted from cells according to the manufacturer's instructions. The concentration of total RNA was determined by Nanodrop (Thermo Scientific, USA), and cDNA was generated using EvoM-MLVRT Master Mix (Accurate Biology, China).

RT-PCR was performed using 2 × Taq Master Mix (Vazyme, China), and the reaction system and procedures were conducted in terms of the manufacturer's manual. Electrophoresis of PCR products was performed on a 2% agarose gel, and images of bands were acquired by a chemiluminescence imaging system (ChampGel 5000, China).

The qPCR was conducted utilizing the SYBR Green Premix Pro TaqHs qPCR kit (Accurate Biology) on a Bio-Rad CFX96 system. *ACTB* was utilized as a housekeeping gene, and the $2^{-\Delta\Delta}$ cycle threshold (Ct) method was employed to determine mRNA expression level. Primer sequences for selected genes were shown in Table S4.

Immunocytochemistry and immunohistochemistry

Cells were collected for cytospin slides with cold PBS and fixed in 4% PFA for 15 min. After three washes in cold PBS, cells were permeabilized with 0.25% Triton X-100 (Sigma) in PBS for 15 min. The cells were blocked with 5% BSA for 1 h at room temperature, and they were incubated with primary antibodies overnight at 4 °C. Cells were washed three times with PBS and incubated for 1 h with Alexa Fluor 488-conjugated IgG or Alexa Fluor 594-conjugated IgG secondary antibodies. Cell nuclei were stained with DAPI for 7 min, and images were captured under a fluorescence microscope (Leica DM3000, Germany).

For the immunohistochemistry, testis sections were deparaffinized with xylene and hydrated with graded ethanol. Endogenous peroxidase was inactivated with H_2O_2, and antigen retrieval was conducted with 1X sodium citrate buffer for 10 min. Permeabilization was performed with 0.25% Triton X-100 for 15 min, and the sections were blocked with 5% BSA for 1 h. Testis sections were incubated with primary antibodies overnight at 4 °C. The sources and dilutions of antibodies were shown in Table S2. Sections were washed with PBS, incubated with HRP-labeled secondary antibody for 1 h at room temperature, and visualized with a DAB kit. The nuclei of cells were stained with hematoxylin. For immunofluorescence, sections were incubated with Alexa Fluor conjugated secondary antibodies for 1 h at room temperature, and cellular nuclei were stained with DAPI. Images were captured by a Leica microscope.

Western blots

Cells were lysed with RIPA lysis buffer (Beyotime, China) for 30 min on ice and centrifuged at 12,000 rpm for 30 min at 4 °C. Fifty micrograms of total protein from each sample were separated on SDS-PAGE gels and transferred to polyvinylidene difluoride (Millipore, Germany). Membranes were blocked with QuickBlock blocking buffer (Beyotime, China), and they were incubated with primary antibodies overnight at 4 °C and followed by horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Antibodies and dilution ratios were shown in Table S2.

Images were captured by a Mini chemiluminescence imaging system.

CCK-8 assay

The proliferation potential of SLCs after transfection with WNT5A siRNAs or vectors was assessed with a CCK-8 kit (Dojindo, Japan). Cells were seeded into 96-well plates and cultured for 5 days. The complete medium was added with 10% CCK-8 reagent, and cells were incubated for 3 h. The absorbance (OD values) of cells at 450 nm was measured with a microplate reader (Thermo Scientific).

EdU Incorporation assay

EdU (RiboBio) Reagent A and DMEM/F12 were diluted with a ratio of 1:4,000. The cells were incubated with EdU for 16 h, fixed in 4% PFA for 10 min, and neutralized with 2 mg/mL glycine for 7 min. Permeabilization was performed with 0.5% Triton X-100 in PBS for 10 min. Staining was conducted with Apollo reaction solution for 30 min in the dark, and cell nuclei were stained with DAPI for 7 min. Images were acquired with a fluorescence microscope (Leica DMi8, Germany) to calculate the percentages of EdU-positive cells.

Flow cytometry

SLCs were washed twice with PBS, and 10^6 cells were resuspended in Annexin V buffer (BD Biosciences, USA) pursuant to the manufacturer's instructions. In brief, cells were incubated with 5 μL Annexin V-APC and 10 μL propidium iodide (PI) solution for 15 min at room temperature in the dark. The apoptosis of SLCs was detected by FACS Canto II flow cytometry (BD Biosciences).

TUNEL assay

Apoptosis of SLCs was also determined using the Tunel Cell Death Detection Kit (Roche, Switzerland) according to the manufacturer's instructions. Cells were treated with 20 μg/mL proteinase K for 5 min and incubated with dUTP-labeled/terminal deoxynucleotidyl transferase (TdT) enzyme buffer for 1 h in the dark, and cellular nuclei were stained with DAPI. Images were captured with a fluorescence microscope (Leica DMi8, Germany).

Testosterone assay

For detecting testosterone concentration, LCs' differentiation of SLCs was conducted with the induction medium containing phenol red-free DMEM/F12 (Gibco), 2% FBS (Gibco), 0.5 μM Smoothened agonist (Millipore), 2 ng/ml LH (R&D), 1 nM thyroid hormone (PeproTech), 10 ng/ml PDGFBB (PeproTech), 70 ng/ml insulin-like growth factor 1 (PeproTech), 1 ng/ml luteinizing hormone (Sigma), and 1 × ITS (Gibco). Cell supernatants were collected at days 0, 2, 4, 8, and 12 of culture, and testosterone concentrations in cell culture supernatants were measured using the ELISA kit (R&D) in terms of the manufacturer's instructions.

RNA sequencing

RNA sequencing and analysis were performed by Genenergy Co., Ltd (Shanghai, China). Total RNA was isolated using Trizol (Invitrogen), and RNA quality was determined by Nanodrop (Thermo Scientific). RNA integrity was checked by agarose gel electrophoresis. Libraries were constructed using TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, USA) according to the manufacturer's instructions. Libraries were sequenced with the Illumina HiSeq XTen platform to generate 150 bp paired-end reads. Differential gene expression analysis was conducted using the DESeq (2012) R software package, while $P < 0.05$ and fold change ≥ 2 were set as thresholds for the differentially expressed genes (DEGs). Hierarchical cluster analysis of DEGs was performed to demonstrate the gene expression patterns of different groups, and DEGs were analyzed by Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG).

Single cell RNA-sequencing analysis

Cell cluster analysis was performed with the Seurat package on five normal testis samples from the scRNA-seq dataset GSE149512 of the adult testis. The feature table, barcode table, and matrix table into R with the Read10X function were loaded, and the creation, normalization, and scale of Seurat objects from these data were performed using default settings. Specifically, poor-quality cells were filtered out and standard data processing procedures were performed at the same time. Cells were filtered by the following threshold parameters: total number of expressed genes 500 -9,000, total number of UMIs between $-\infty$ and 35,000, and proportion of mitochondrial gene expression < 40%, in terms of the package manual (https://satijalab.org/seurat/v3.1/pbmc3k_tutorial.html) for gene normalization. Cells without NGFR or PDGFRA expression were filtered out, and the remaining cells were clustered again. Uniform manifold approximation and projection (UMAP) and cluster analysis were further conducted. Seurat FindAllMarkers function (test. use = Wilcox) to identify unique cluster-specific marker genes based on the normalized UMI counts. Unless otherwise stated, only genes detected in at least 10% of cells were tested and a threshold of $P < 0.05$ and fold change ≥ 2 or ≤ -2 was set for the DEGs. The genes associated with the cell surface

and with the highest proportion of cell clusters were chosen from the differential test table using GO: 0009986. Single-cell pseudotime developmental trajectories were performed according to the Operator Manual (http://cole-trapnell-lab.github.io/monocle-release/docs_mobile/) using the Monocle 2 software package (version 2.8.0).

Statistical analysis

Statistical analysis was performed with GraphPad Prism 8.0. Each experiment was repeated three times, and all data were presented as mean ± SD. Statistical differences between two groups were calculated using the *t*-test, and $P < 0.05$ was considered statistically significant. * indicated $P < 0.05$, while ** denoted $P < 0.01$.

Results

Screening for specific cell surface markers that identify and isolate human SLCs

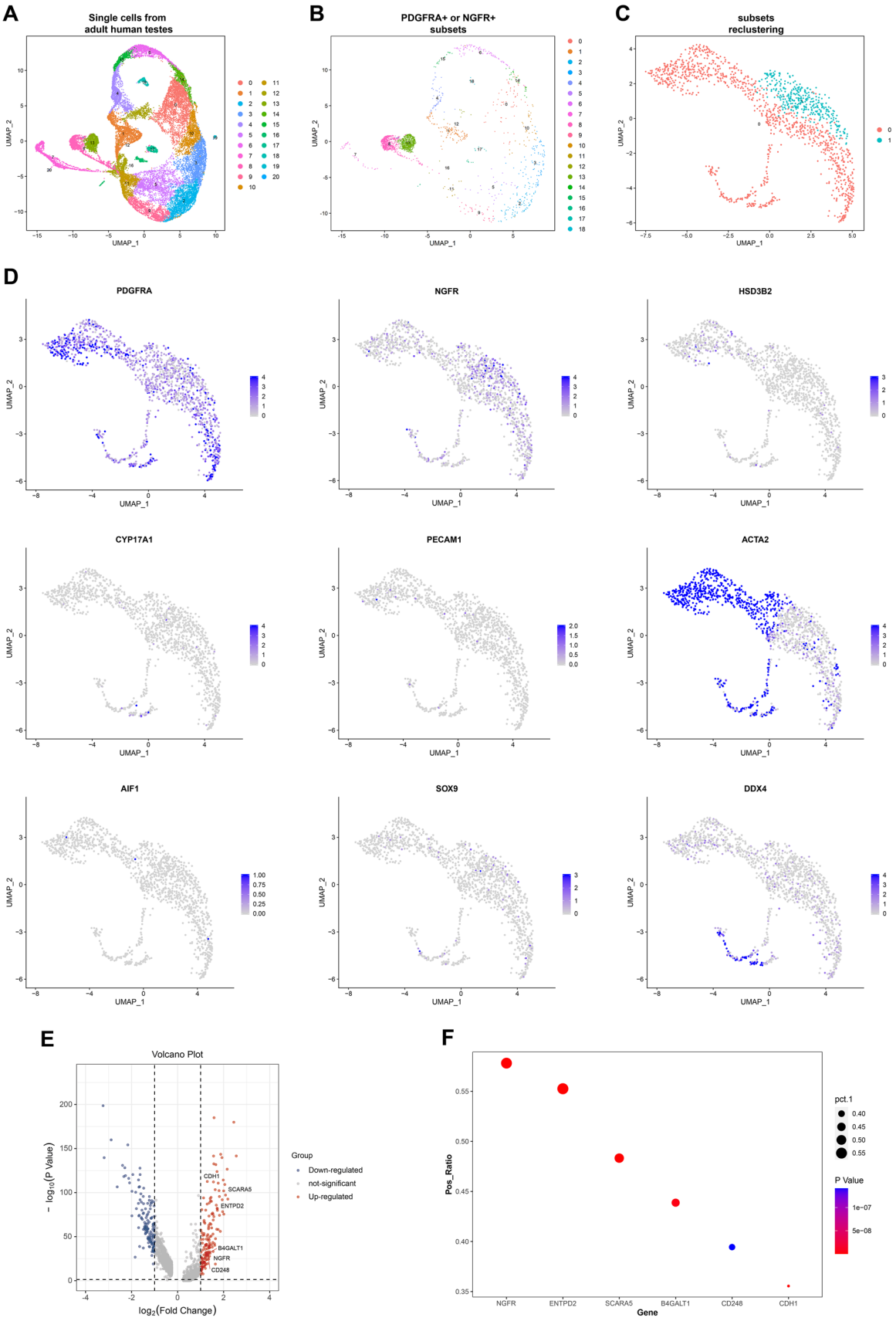
To select specific cell surface markers that could identify and isolate human SLCs, we first analyzed the UMAP profiles of single-cell transcriptome data GSE149512 containing a total of five normal spermatogenesis testis tissues from adults, and 46,453 features and 26,079 cells remained after data filtering (Fig. 1A). Next, we filtered out cells without PDGFRA or NGFR expression (Fig. 1B). Interestingly, our cluster analysis revealed two major subpopulations (Fig. 1C, D). Testicular tissues mainly contained three germ cell clusters and five somatic cell clusters. Cluster 0 and Cluster 1 showed negligibly positive rates for HSD3B2, CYP17A1, PECAM1, AIF1, SOX9, or DDX4, indicating that they were not differentiated LCs, endothelial cells, macrophages, Sertoli cells, or germ cells. NGFR was predominantly expressed in Cluster 1, and ACTA2 was mainly present in Cluster 0, reflecting that Cluster 1 was putative human SLCs and Cluster 0 contains peritubular myoid cells (Fig. 1D). Further dimensionality reduction clustering revealed that the putative SLCs in Cluster 1 could be divided into three subsets and the developmental trajectories by pseudotime, and the accuracy of the developmental trajectories was further identified by the expression and positive rates of the common mature SLCs markers NGFR, CD248, PDGFRA, and THY1 (Figure S1A). Differential gene enrichment was employed to identify the marker genes in each Cluster. Finally, a total of 310 DEGs were identified in Cluster 0 and Cluster 1, with 161 upregulated genes and 149 downregulated genes in Cluster 1 (Fig. 1E). In total, 161 upregulated genes in Cluster 1 were analyzed by GO analysis, and the genes associated with the cell surface (GO: 0009986) and with the highest positive expression rate in Cluster 1 cells were selected (Figure S1B). The proportion of $NGFR^+$ cells was found to be the highest in Cluster 1, indicating that NGFR may be the most suitable marker for isolating and purifying human SLCs (Fig. 1F).

Isolation and characterization of human SLCs

To obtain and characterize $NGFR^+$ cells, human testis cells were sorted using fluorescence-activated cell sorting (FACS), and $NGFR^+$ cells represented 0.41% of the total LCs population (Fig. 2A). These freshly isolated $NGFR^+$ cells were then cultured at a 1×10^5/ml density in dishes supplemented with a complete expansion medium. Adherent P0 passage $NGFR^+$ cells showed typical cellular morphology of SLCs, which was clearly distinguishable from non-SLCs (Fig. 2B). RT-PCR and immunocytochemistry showed that the isolated $NGFR^+$ cells expressed NGFR (94.82%), PDGFRA (95.20%), NES (89.11%), NR2F2 (96.41%), and THY1 (92.88%) (Fig. 2 C, E and G), hallmarks for human SLCs rather than HSD3β, a marker of the differentiated LCs. In addition, NGFR-negative cells expressed LHCGR (98.46%), SF-1 (94.75%), STAR (92.71%), HSD3β (96.34%), and CYP17A1 (88.65%) (Fig. 2 D, F and H), markers for the differentiated LCs but not NGFR, a marker of SLCs. These results demonstrate that NGFR could be an excellent hallmark for SLCs in humans.

WNT5A is predominantly expressed in human SLCs and it is involved in regulating stem cell-related function

To explore novel genes that regulate the biological function of human SLCs, we performed RNA sequencing to compare the global transcriptional profiles of the validated human SLCs and non-SLCs. A total of 34,645 genes were detected in these two types of cells. Compared to non-SLCs, 6,346 DEGs were found in SLCs compared to non-SLCs, with 2,673 in upregulated genes and 3,673 downregulated genes (Fig. 3A and B). The upregulated genes with fold changes in the top 100 were selected for GO and KEGG analyses. Most DEGs were found to be mainly involved in biological processes, e.g., cell proliferation, migration, localization, adhesion, and cycle, which were primarily regulated by signaling molecules, e.g., WNT, TNF, ECMR, and PI3K (Figure S2). We selected the top 30 upregulated genes associated with stem cell function for qPCR validation, and we found that the trend of all DEGs was consistent with RNA-sequencing results (Fig. 3C). Notably, we found that WNT5A was one of the genes that were most strongly associated with stem cell function in these DEGs, and KEGG analysis also showed enrichment of the WNT signaling pathway. Therefore, WNT5A was chosen for further protein expression validation, and Western blots revealed that WNT5A expression


A
Single cells from adult human testes
B
PDGFRA+ or NGFR+ subsets
C
subsets reclustering
D
PDGFRA
NGFR
HSD3B2
CYP17A1
PECAM1
ACTA2
AIF1
SOX9
DDX4
UMAP_1
UMAP_2
E
Volcano Plot
Group
Down-regulated
not-significant
Up-regulated
CDH1
SCARA5
ENTPD2
B4GALT1
NGFR
CD248
log2(Fold Change)
− log10(P Value)
F
Pos_Ratio
pct.1
P Value
Gene
NGFR
ENTPD2
SCARA5
B4GALT1
CD248
CDH1

◂**Fig. 1** Single cell RNA-sequencing (scRNA-seq) data analysis of cell surface marker gene expression in the putative human SLCs. **A** UMAP plots of adult testis cell clusters by scRNA-seq analysis. **B** PDGFRA$^+$ or NGFR$^+$ subsets screened from panel (**A**). **C** The UMAP plot was generated from the subsets defined in **B** by clustering analysis. **D** Expression patterns of markers in different types of cells within the testis in cluster defined in (**C**). SLCs' hallmarks: PDGFRA and NGFR, LCs' markers: HSD3B2 and CYP17A1, endothelial cell marker PECAM1, myoid cell marker ACTA2, macrophage marker AIF1, Sertoli cell marker SOX9, and germ cell marker DDX4. Blue indicated high expression, and gray indicates low or no expression. **E** Volcano plots showed the DEGs identified in cluster 0 and cluster 1. Red dots indicated the upregulated genes, whereas blue dots denoted the downregulated genes in cluster 1. **F** The cell surface marker expression pattern from the differential expression cluster 1 was shown in (**C**). The dot colour represented the *P*-value, and the dot size indicated the percentage of expression

level was significantly upregulated in SLCs compared with non-SLCs (Fig. 3D and E). We then determined WNT5A expression in human testicular tissues with normal spermatogenesis and found that WNT5A was predominantly expressed in the Leydig cells (Fig. 3F–H). Specific clinical characteristics of patients with testicular tissues were presented in Table S1. Next, we examined the subcellular localization of WNT5A in the human testes, and immunohistochemistry illustrated that WNT5A was colocalized with NGFR (arrows) or NES (arrows) in human SLCs (Fig. 3I). In addition, we performed double immunostaining of the isolated SLCs, and we revealed coexpression of WNT5A and NGFR in nearly all SLCs (Fig. 3J). Collectively, these data indicate that WNT5A is mainly expressed in human SLCs and it might play a vital role in regulating the biological function of human SLCs.

WNT5A knockdown inhibits the proliferation and DNA synthesis of human SLCs

To investigate the function of WNT5A in regulating fate determinations of human SLCs, WNT5A knockdown of human SLCs was performed by siRNAs. The transfection efficiency of siRNAs in human SLCs was 82.23% as illustrated by Cy3-labeled siRNA transfection (Fig. 4A and B). Our qRT-PCR and Western blots showed that all three siRNAs could significantly decrease the expression levels of WNT5A, while WNT5A-siRNA1 and WNT5A-siRNA3 assumed relatively better silencing effect (Fig. 4C, D, E). Next, we found that WNT5A-siRNAs significantly inhibited the expression level of PCNA, a marker of cell proliferation, as detected by Western blots (Fig. 4F and G). CCK-8 assay displayed that WNT5A-siRNAs remarkably diminished the proliferation ability of human SLCs from day 2 compared to the control siRNA (Fig. 4H). We used EdU incorporation assays to examine DNA synthesis in human SLCs. Compared to control siRNA, EdU-positive cells were obviously decreased at 72 h after transfection with WNT5A-siRNA1 and WNT5A-siRNA3 (Fig. 4I and J). Considered together, the above data indicate that WNT5A silencing leads to decreases in the proliferation and DNA synthesis of human SLCs.

The knockdown of WNT5A enhances the apoptosis of human SLCs

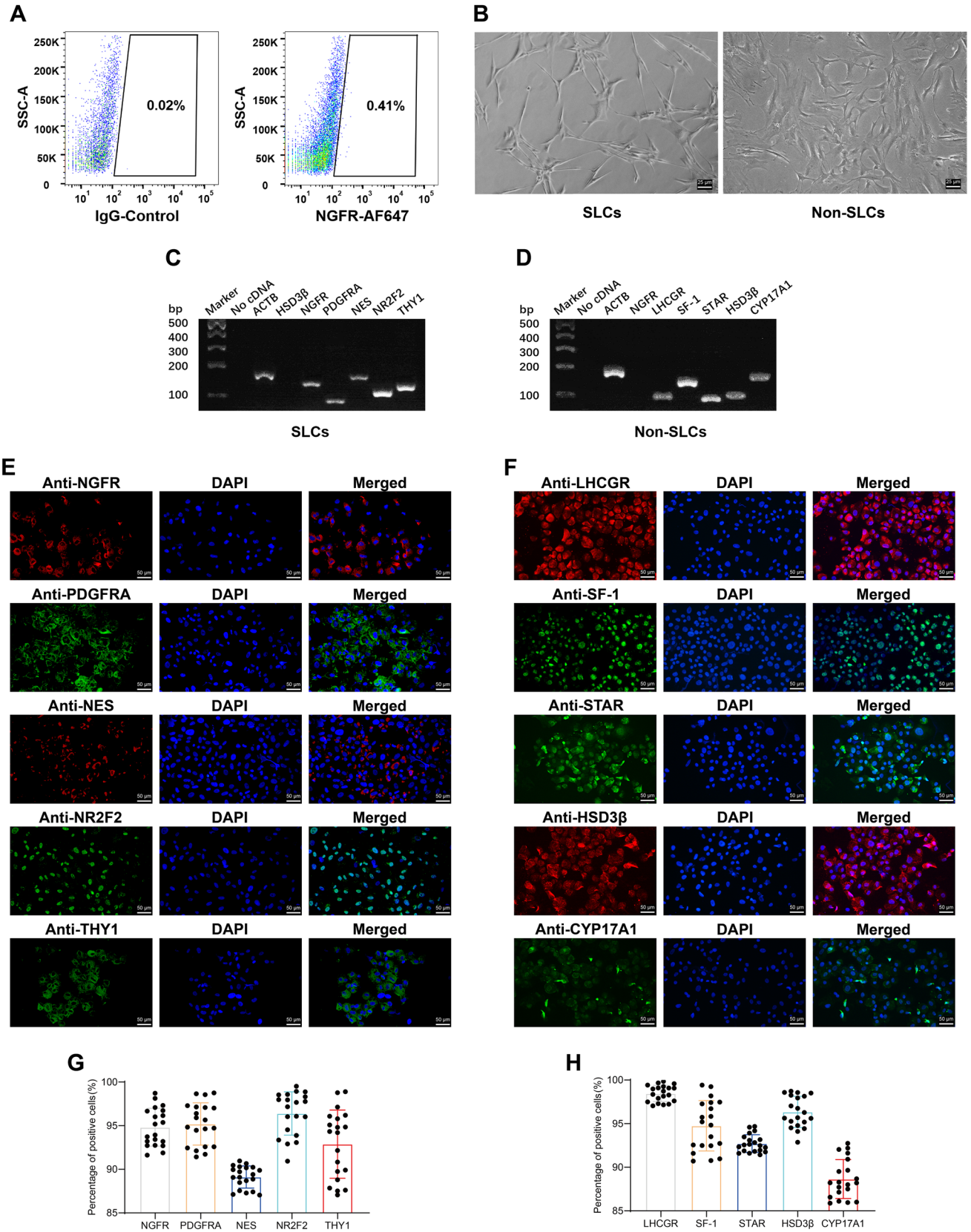
We conducted TUNEL assay, Annexin V/PI staining and flow cytometry to examine the impact of WNT5A knockdown on human SLCs' apoptosis. Our flow cytometric analysis revealed a significant increase in both early and late apoptosis of human SLCs after 72 h of treatment with WNT5A siRNA1 and WNT5A siRNA3 when compared to the control siRNA (Fig. 5A–C). TUNEL assay results were consistent with flow analysis, with a higher percentage of TUNEL-positive cells in WNT5A siRNA1 and WNT5A siRNA3 than the control siRNA (Fig. 5D and E). Together, these findings suggest that WNT5A knockdown enhances the apoptosis of human SLCs.

WNT5A knockdown causes inhibition of stemness in human SLCs

To further investigate the role of WNT5A in regulating stemness of human SLCs, we examined changes in expression levels of stemness-related marker proteins in human SLCs. Western blots showed that WNT5A siRNA1 and WNT5A siRNA3 significantly decreased the expression levels of NGFR, NES, NR2F2, and THY1, markers for SLCs, compared with control siRNA at 72 h after transfection (Fig. 5 F and G). Based upon these data, it can be concluded that WNT5A is involved in maintaining the stemness of human SLCs.

WNT5A knockdown causes an increase in LCs' lineage differentiation of human SLCs

To assess the effect of WNT5A on the ability of SLCs to differentiate into LCs in vitro, we employed the differentiation medium for human SLCs after transfection with WNT5A siRNA3 and harvested culture supernatants for testosterone assays at indicated time points. We observed that SLCs in the undifferentiated state could not secrete testosterone. Interestingly, testosterone production could be detected at day 8 of the induced differentiation of human SLCs, and testosterone synthesis was gradually increased at day 12 (Fig. 5H). More importantly, testosterone synthesis was significantly increased in induced differentiation of human SLCs by treatment with WNT5A siRNA3 compared to control siRNA (Fig. 5H). Taken together, these results implicate that human SLCs can differentiate in vitro into LCs capable of producing testosterone and that WNT5A knockdown


A
SSC-A
0.02%
IgG-Control
0.41%
NGFR-AF647
B
SLCs
Non-SLCs
C
bp
Marker
No cDNA
ACTB
HSD3β
NGFR
PDGFRA
NES
NR2F2
THY1
SLCs
D
bp
Marker
No cDNA
ACTB
NGFR
LHCGR
SF-1
STAR
HSD3β
CYP17A1
Non-SLCs
E
Anti-NGFR
DAPI
Merged
Anti-PDGFRA
Anti-NES
Anti-NR2F2
Anti-THY1
F
Anti-LHCGR
Anti-SF-1
Anti-STAR
Anti-HSD3β
Anti-CYP17A1
G
Percentage of positive cells(%)
NGFR
PDGFRA
NES
NR2F2
THY1
H
LHCGR
SF-1
STAR
HSD3β
CYP17A1

◂**Fig. 2** Isolation and characterization of human SLCs. **A** $NGFR^+$ cells were isolated from adult testicular LCs by FACS. **B** Representative phase-contrast micrograph of putative $NGFR^+$ SLCs and non-SLCs at P0. **C**, **E** RT-PCR and immunocytochemistry showed that $NGFR^+$ cells expressed NGFR, PDGFRA, NES, NR2F2 and THY1 but not HSD3β. **D**, **F** $NGFR^-$ cells expressed LHCGR, SF-1, STAR, HSD3β and CYP17A1 rather than NGFR. **G**, **H** Percentages of $NGFR^+$ cells and $NGFR^-$ cells. Scale bars in **E** and **F** = 50 μm

results in an enhancement in LC lineage differentiation of human SLCs.

WNT5A knockdown reverses the effect of WNT5A overexpression in human SLCs

To further elucidate the regulation of WNT5A in human SLCs, WNT5A siRNA3 and pCMV-WNT5A overexpression plasmids were cotransfected into human SLCs. Our qRT-PCR and Western blots showed that WNT5A siRNA3 decreased the levels of WNT5A, whereas oeWNT5A enhanced the levels of WNT5A (Figure S3). In contrast to pCMV-WNT5A overexpression, CCK-8 assay showed that cotransfection of WNT5A siRNA3 and oeWNT5A significantly reversed the proliferation of human SLCs (Fig. 6A). Cotransfection of WNT5A siRNA3 and oeWNT5A reduced the percentage of EdU-positive cells in human SLCs compared to pCMV-WNT5A overexpression (Fig. 6B and C). In addition, WNT5A siRNA3 and oeWNT5A increased apoptotic cells in human SLCs compared with pCMV-WNT5A overexpression (Fig. 6D and E). Western blots showed that the expression levels of NGFR, NES, NR2F2, and THY1, were significantly reduced by WNT5A siRNA3 and oeWNT5A in human SLCs compared with pCMV-WNT5A overexpression (Fig. 6F and G). Taken together, these data further demonstrate an important role for WNT5A in regulating proliferation, DNA synthesis, apoptosis, and stemness in human SLCs.

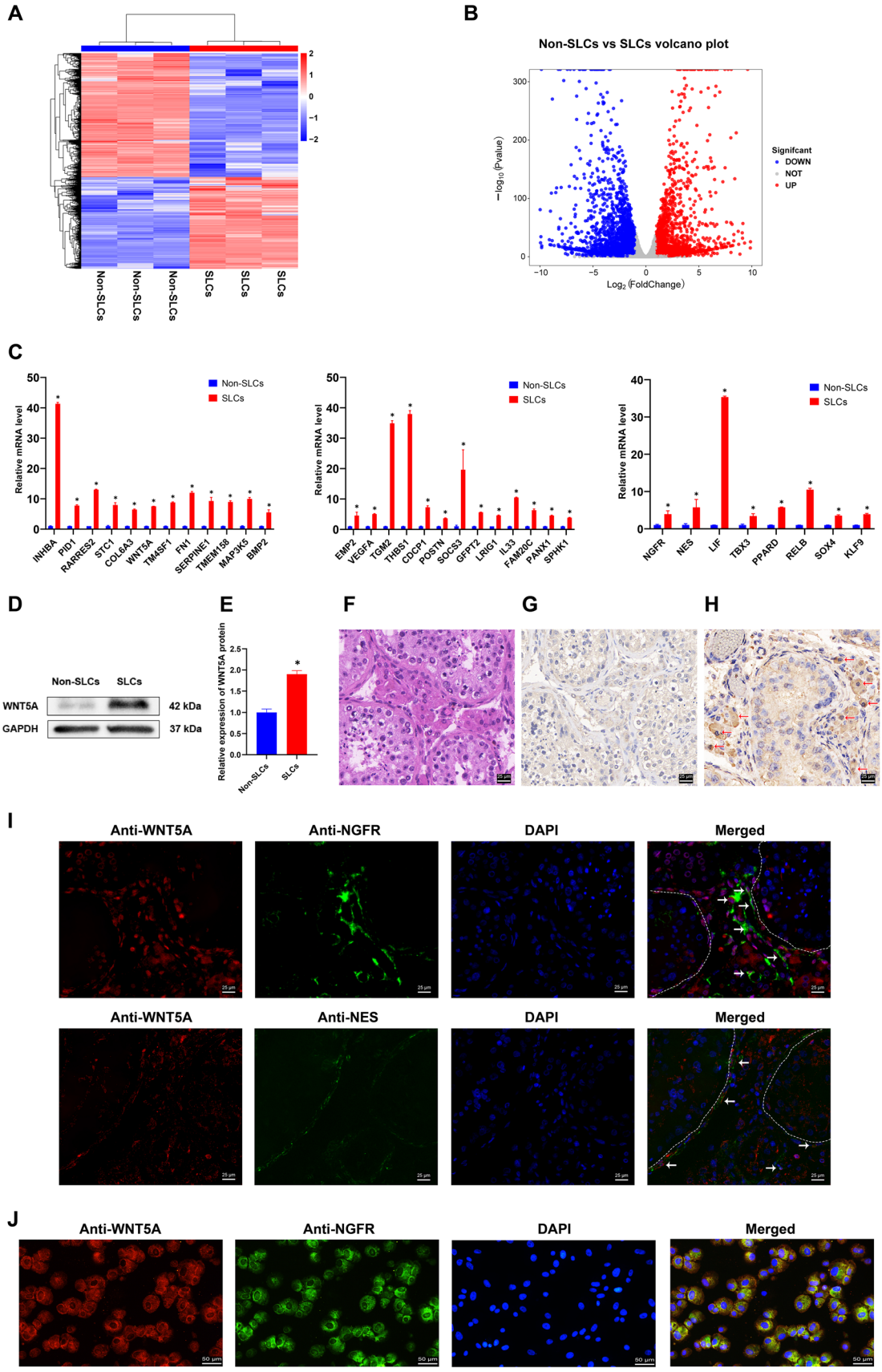
WNT5A activates β-catenin signaling in human SLCs

To further explore the potential mechanism by which WNT5A regulates SLCs, we determined the β-catenin signaling pathway, which is critical for stem-like characteristics, in WNT5A silencing SLCs compared to control siRNA. We examined β-catenin expression levels in whole cell, cytoplasmic, and nuclear extracts, essential players of the WNT5A/β-catenin signaling pathway. Western blots showed that WNT5A knockdown resulted in a significant decrease in β-catenin expression levels in whole cells and cell nuclei compared to control siRNA (Fig. 7A and B), whereas cytoplasmic β-catenin expression was not significantly different between WNT5A siRNAs and control siRNA (Fig. 7A and B). Similarly, immunocytochemistry displayed that WNT5A knockdown led to fewer positive cells of β-catenin in the nuclei compared to control siRNA (Fig. 7C and D). Finally, we examined the expression levels of β-catenin pathway-related proteins. Western blots displayed that WNT5A knockdown resulted in a significant reduction of c-Myc, CD44, and Cyclin D1 protein expression levels compared with control siRNA (Fig. 7E and F). Taken together, these results implicate that WNT5A contributes to the nuclear translocation of β-catenin and β-catenin signaling pathway activation, which is related to the stemness of human SLCs.

Discussion

Studies on human SLCs have been challenged due to the difficulty in obtaining human testicular tissues and lacking of effective methods for identifying and isolating human SLCs. Nevertheless, with emerging single-cell sequencing technologies, it is feasible to analyze global transcriptome data from adult testis and map transcriptional signatures of putative human SLCs. By isolating $NGFR^+$ LCs with proliferative potential, we demonstrated that these cells had SLCs phenotype. Thus, NGFR could be employed as an excellent marker for identifying and isolating human SLCs, which makes it possible to unveil the molecular mechanisms that regulate the fate determinations of human SLCs.

It has recently been reported that WNT5A can induce M2 polarization of TAM by regulating IL-10 secretion through the CaKMII-ERK1/2-STAT3 pathway, which ultimately promotes the proliferation and migration of colorectal cancer cells [38]. Additionally, it has been shown that WNT5A stimulates ERK1/2 activation and enhances chronic lymphocytic leukemia cell proliferation via a ROR1/DOCK2-dependent pathway [39]. Similarly, WNT5A has been found to promote chondrocyte proliferation, hypertrophy, and migration by controlling c-MYC and Cyclin D1 expression and transcriptional activity [40]. Although WNT5A plays a role in many normal tissue development and diseases, its function and mechanism in mediating the development of human Leydig cell lineages remain unknown. In this study, we have demonstrated that WNT5A knockdown significantly inhibited the proliferation and DNA synthesis of human SLCs as well as both early and late apoptosis of these cells via the β-catenin signaling pathway. This discovery could aid in expanding human SLCs in vitro, ensuring that there are enough human SLCs available for basic research and translational applications. This finding can be supported by a previous study showing that inhibition of β-catenin signaling leads to an increase in apoptosis and damage to articular cartilage in Col2a1-ICAT transgenic mice. These results and our data indicate that the WNT/β-catenin signaling pathway plays an anti-apoptotic role [41]. WNT7A has been found to trigger dedifferentiation and prevent apoptosis in primary articular chondrocytes by activating PI3k and Akt pathways,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CDCP1
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FAM20C
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◂**Fig. 3** WNT5A regulates SLCs function and the expression and cellular localization of WNT5A in human testis. **A** Hierarchical clustering showed the DEGs between human SLCs and non-SLCs. **B** Volcano plot illustrated the DEGs between human non-SLCs and SLCs. **C** The qPCR was performed to assess the expression changes of the top upregulated 30 genes associated with stem cell function in human SLCs. **D**, **E** Western blots showed the changes in WNT5A expression in human SLCs and non-SLCs. **F** H&E staining of human testis tissues with normal spermatogenesis. **G** Immunohistochemistry was used to detect the cellular localization of normal IgG in human testis. **H** Immunohistochemistry revealed cellular localization of WNT5A in human testis. Arrows indicated WNT5A-positive cells in human testes. **I** Double immunostaining showed colocalization of WNT5A with NGFR and WNT5A with NES in normal human testes. **J** Double immunostaining illustrated coexpression of WNT5A with NGFR in human SLCs. Scale bars in **F**, **G**, **H**, and **I** = 25 μm, scale bars in **J** = 50 μm. Specific clinical characteristics of NOA patients with testicular tissues were presented in Table S1

and WNT7A inhibits nitric oxide-induced apoptosis, which reflects that WNT has anti-apoptotic properties [42].

It has previously been reported stemness markers in human SLCs, including NGFR, Nestin, and PDGFRA [18–20]. Rodent SLCs stemness markers, namely NR2F2 and THY1, have been found to be expressed in human SLCs [43]. Here, we have demonstrated that WNT5A silencing led to the reduction in expression levels of stem cell-related markers, including NGFR, NES, NR2F2, and THY1, in human SLCs, which implies that WNT5A is involved in maintaining the stemness of human SLCs. Similar to our results, WNT5A-dependent activation of WNT/β-catenin signaling has been shown to promote colony formation, self-renewal, and T cytokine/lymphoenhancer binding factor (TCF/LEF) transcriptional activity in endometrial mesenchymal stem cells [44]. Additionally, WNT5A may stimulate spermatogonial stem cell self-renewal through the β-catenin-independent mechanism [35]. The expression level of PDGFRA was increased by WNT5A siRNAs in human SLCs. It has been reported that PDGFRA is present in both undifferentiated SLCs and differentiated LCs and it may be expressed at a higher level in differentiated LCs [13, 45, 46]. Our results showed that WNT5A silencing caused suppression of stemness in human SLCs and enhanced the ability of human SLCs to enter LCs' lineage differentiation. The combined effect of these two aspects leads to the transition of SLCs to differentiated LCs, which results in an increase in expression of PDGFRA. More importantly, our testosterone concentration assays further support the finding that fate regulation of stemness and differentiation of human SLCs by WNT5A silencing leads to a significant increase in testosterone synthesis after differentiation.

The WNT/β-catenin pathway is primarily responsible for regulating cell fate determinations during development [47]. When WNT ligands bind to their receptors, WNT signaling is initiated, and β-catenin separates from the destruction complex, namely Axin, APC, and GSK3β. β-catenin then moves to cellular nuclei, where it binds to the coactivator TCF/LEF to form a transcriptional complex that regulates the expression of target genes, e.g., c-Myc, CD44, and cyclin D1 [32]. It has been shown that the activation of the WNT pathway and nuclear translocation of β-catenin are closely related to cell proliferation and tumorigenesis [48, 49]. IRF3 knockdown abnormally activates the Wnt signaling pathway and promotes β-catenin nuclear translocation, thereby promoting intestinal epithelial cell proliferation [50]. NU2058 inhibits the transcription of c-Myc and cyclin D1 by promoting the nuclear export of β-catenin, which in turn leads to cellular senescence [51]. Consistent with these findings, we have demonstrated that knockdown of WNT5A resulted in a decrease in β-catenin nuclear expression, thereby affecting the proliferation of SLCs. WNT signaling regulates β-Catenin mainly by phosphorylating downstream proteins [52].

In conclusion, we have reported that NGFR can be identified as an effective marker for isolating human SLCs, and notably, we have demonstrated for the first time that WNT5A mediates proliferation, apoptosis, and stemness of human SLCs through activation of the β-catenin pathway. This study thus provides a novel molecular mechanism regulating fate determinations of human SLCs, and significantly, it could offer a new insight into understanding the human testis niche.

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Author contributions W.L. conducted the experiments and wrote the paper. L.D., Y.C. and C.H. assisted with the experiments and technical advice. Z.H. designed and supervised the laboratory experiments, data analysis and manuscript revisions. All authors approved the manuscript.

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Data availability Request on the data and materials is available from the corresponding author.

Declarations

Conflicts of interest The authors declare no competing interest.

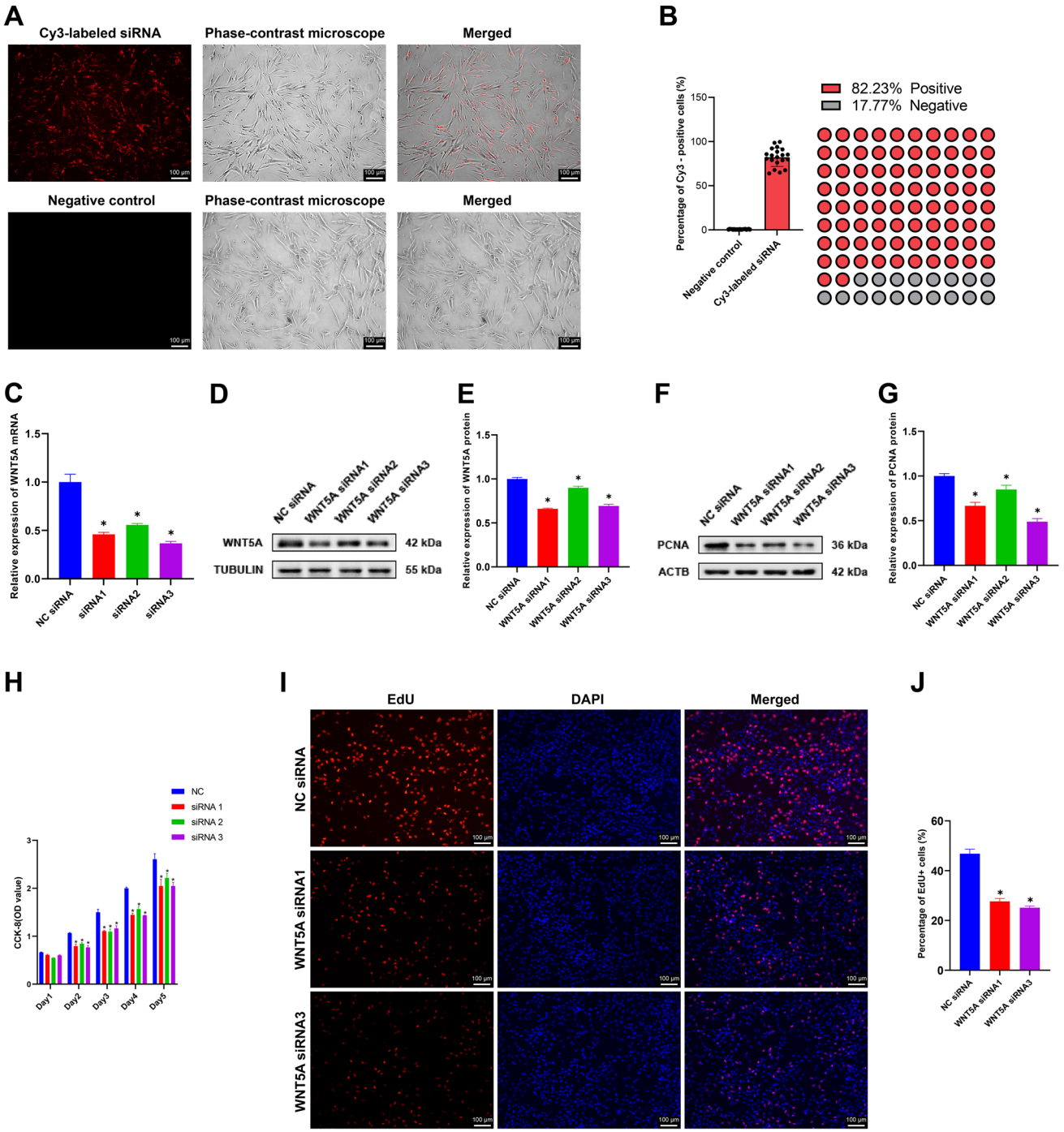

Fig. 4 Effect of WNT5A knockdown on proliferation and DNA synthesis of human SLCs. **A**, **B** Fluorescence microscopy indicated transfection efficiency of WNT5A siRNAs by Cy3-labeled siRNA. **C** The qPCR showed *WNT5A* mRNA levels in human SLCs treated with WNT5A siRNAs and control siRNA. **D**, **E** Western blots displayed protein levels of WNT5A in human SLCs affected by WNT5A siRNAs. **F**, **G** Western blotting showed protein levels of PCNA in human SLCs affected by WNT5A siRNAs. **H** CCK-8 assay revealed the effect of WNT5A siRNAs on the proliferation of human SLCs. **I**, **J** Percentages of EdU-positive cells in human SLCs affected by WNT5A siRNAs

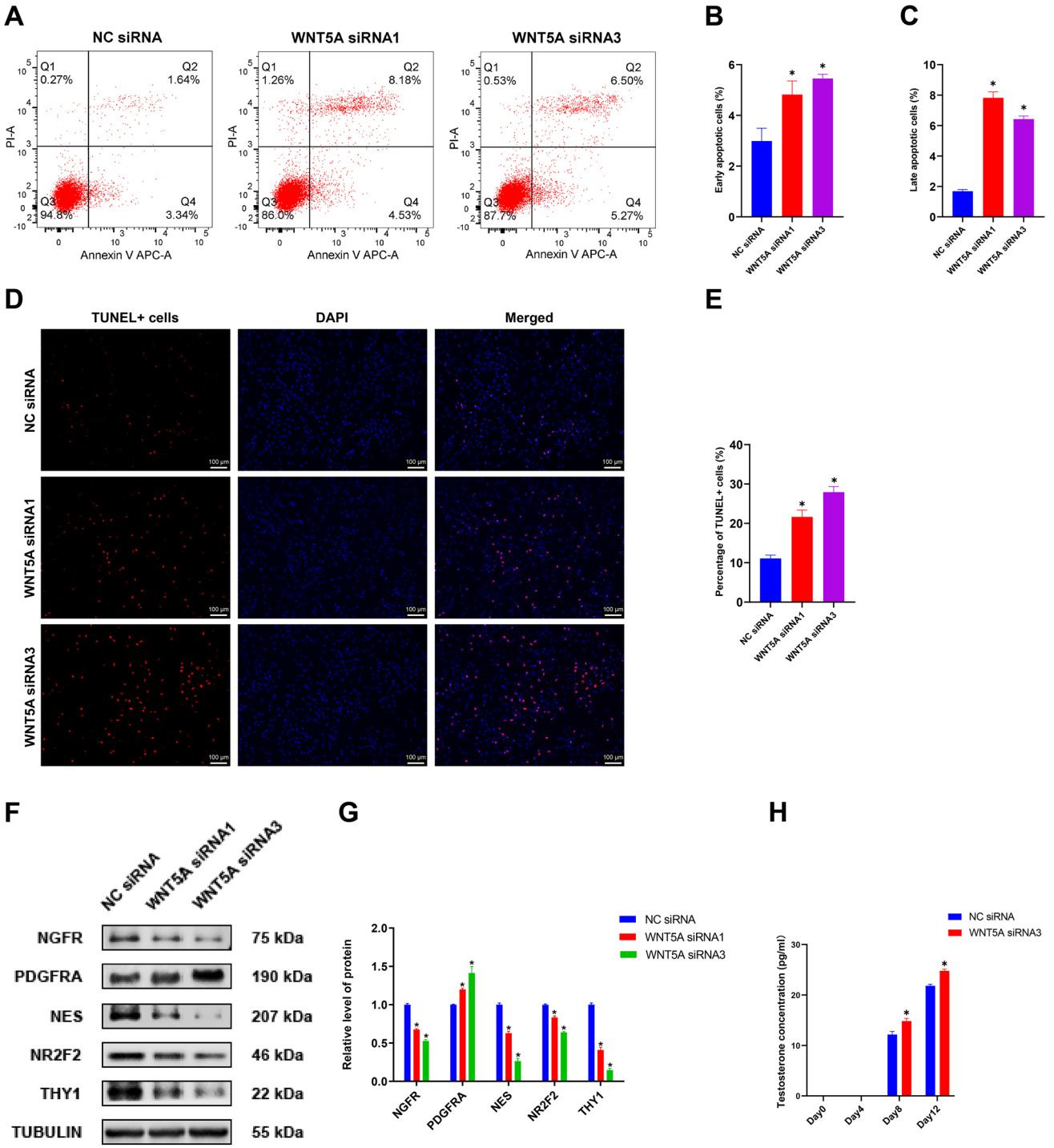

Fig. 5 Effect of WNT5A knockdown on apoptosis, stemness, and lineage differentiation ability in human SLCs. **A–C** Flow cytometry determined the effect of WNT5A siRNAs on the apoptosis of human SLCs. **D**, **E** TUNEL assay measured apoptotic cells of human SLCs when WNT5A was knocked down. **F**, **G** Expression levels of stemness markers of human SLCs when WNT5A was knocked down. **H** Human SLCs were induced to differentiate in vitro to produce testosterone when WNT5A was knocked down

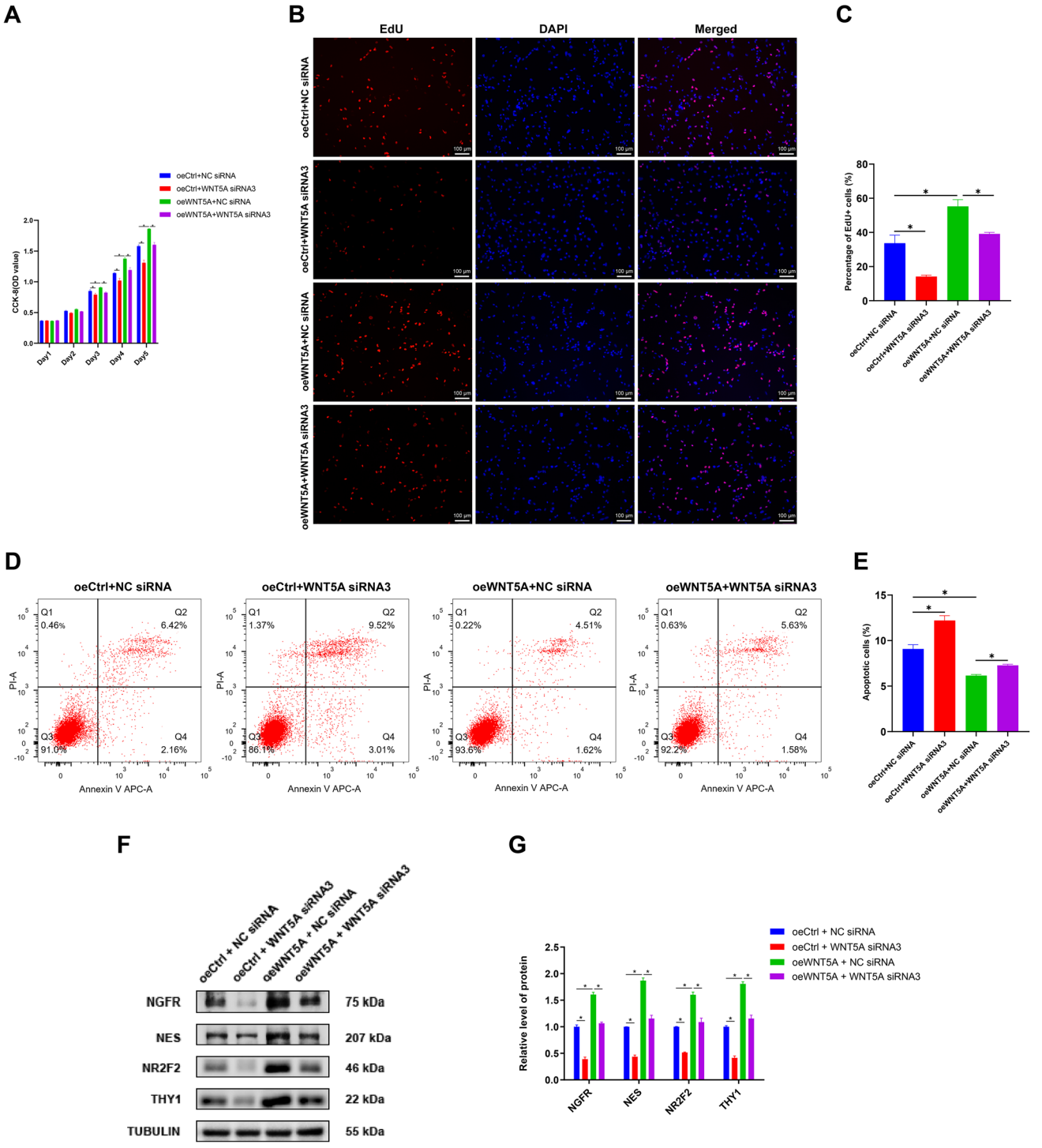

Fig. 6 Effect of WNT5A silencing reversing WNT5A overexpression in human SLCs. **A** CCK-8 assay showed cell proliferation in human SLCs co-transfected with WNT5A siRNA3 and pCMV-WNT5A overexpression (oeWNT5A) plasmids. **B**, **C** Percentages of EdU-positive cells in human SLCs co-transfected with WNT5A siRNA3 and oeWNT5A. **D**, **E** Flow cytometry measured the percentages of apoptosis in human SLCs co-transfected with WNT5A siRNA3 and oeWNT5A. **F**, **G** Western blots examined expression levels of stemness markers in human SLCs co-transfected with WNT5A siRNA3 and oeWNT5A

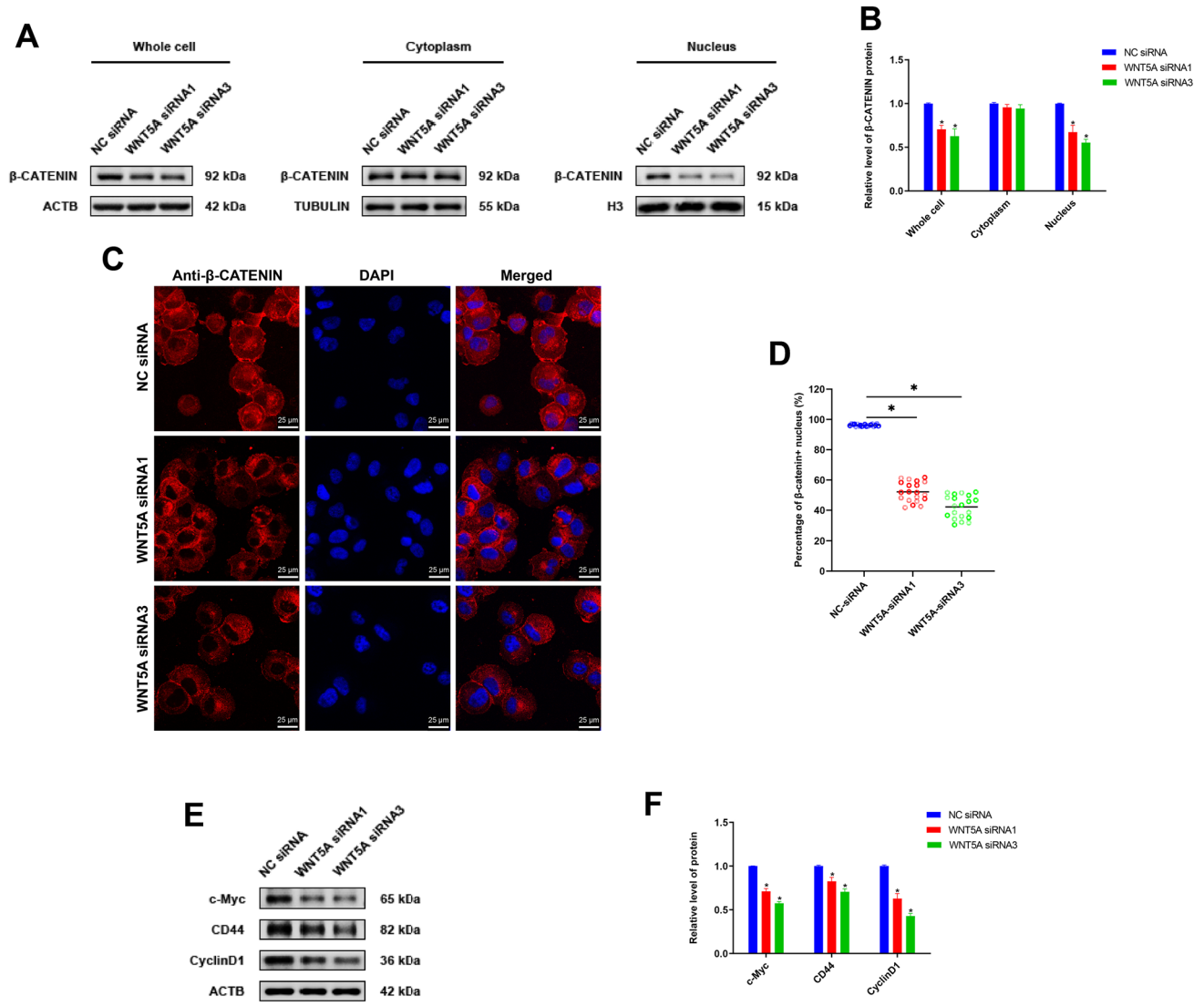

Fig. 7 Influence on the WNT/β-catenin signaling pathway in human SLCs when WNT5A was knocked down. **A**, **B** Western blots revealed β-catenin expression levels in whole cells, cytoplasmic and nuclear extracts of human SLCs affected by WNT5A siRNAs. **C**, **D** Immunofluorescence was used to observe changes in the subcellular localization of β-catenin protein in human SLCs when WNT5A was silenced. **E**, **F** Western blots detected the expression levels of β-catenin pathway-related proteins, including c-Myc, CD44, and cyclin D1, in human SLCs affected by WNT5A siRNAs

Ethics approval and consent to participate This study was approved by the Ethics Review Board of Hunan Normal University, and consent was obtained from human testis tissues of patients used for research only.

Consent for publication All authors agreed to publication of this article.

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