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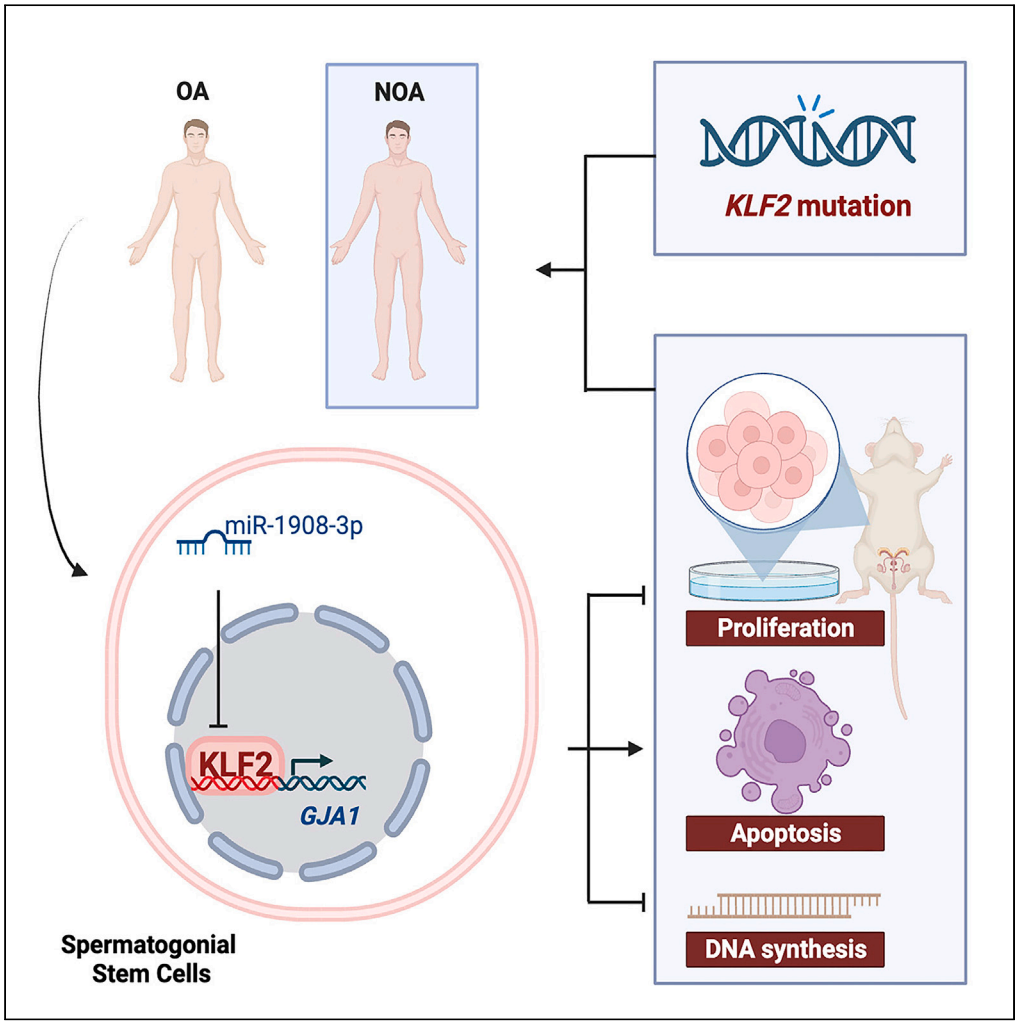

Wei Chen, Yinghong Cui, Chunyun Li, Caimei He, Li Du, Wei Liu, Zuping He

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Highlights

KLF2 mediates proliferation and apoptosis of human spermatogonial stem cells (SSCs)

KLF2 interacts with GJA1 to regulate fate decisions of human SSCs

Abnormalities in high level of KLF2 and/or KIF2 mutations lead to male infertility

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KLF2 controls proliferation and apoptosis of human spermatogonial stem cells via targeting GJA1

Wei Chen,[1] Yinghong Cui,[1] Chunyun Li,[1] Caimei He,[1] Li Du,[1] Wei Liu,[1] and Zuping He[1,2,3,*]

SUMMARY

Human spermatogonial stem cells (SSCs) are essential for spermatogenesis and male fertility. However, molecular mechanisms regulating fate determinations of human SSCs remain elusive. In this study, we revealed that KLF2 decreased the proliferation, DNA synthesis, and colonization of human SSCs as well as increased apoptosis of these cells. We identified and demonstrated that GJA1 was a target gene for KLF2 in human SSCs. Notably, KLF2 overexpression rescued the reduction of proliferation of human SSCs caused by GJA1 silencing as well as the enhancement of apoptosis of human SSCs. Abnormalities in the higher level of KLF2 and/or *KIF2* mutations might lead to male infertility. Collectively, these results implicate that KLF2 inhibits proliferation of human SSCs and enhances their apoptosis by targeting GJA1. This study thus provides novel genetic mechanisms underlying human spermatogenesis and azoospermia, and it offers new endogenous targets for treating male infertility.

INTRODUCTION

Male infertility occurs in 8%–12% of the couple around the world,[1] and notably, it has been increased by 0.291% per year from 1990 to 2017.[2] Increasing evidence indicates that infertile men have a higher risk of cardiovascular disease mortality and cancer morbidity.[3] As such, male infertility has become a major disease that seriously affects human reproductive health and population. Moreover, the problem has gradually become aggravated by numerous factors, e.g., environmental pollution, poor lifestyle, late marriage and childbearing, and COVID-19 pandemic.[4–6] Among them, abnormal spermatogenesis due to testicular dysfunction accounts for approximately 75% of male infertility, and it is the most important cause for this disease.[7]

Spermatogenesis refers to a process by which spermatogonial stem cells (SSCs) self-renew to maintain the pool of stem cells in the testis and differentiate into mature spermatids. It is worth noting that SSCs are the unique adult stem cells that transmit genetic information to offspring, and they are the basis for retaining male fertility. The proliferation and spontaneous apoptosis of SSCs are essential for maintaining normal spermatogenesis in humans. The fate determinations of SSCs are preciously regulated by coding genes and noncoding RNAs. Spermatogenesis-related genes are specifically or predominantly expressed during the process of spermatogenesis and sperm maturation, suggesting that these genes have important effect on controlling male germ cell proliferation, differentiation, and apoptosis. We have recently demonstrated that OIP5 mediates the proliferation and apoptosis of human SSCs via interacting with NCK2.[8] However, the functions and mechanisms of key genes in controlling the proliferation and apoptosis of human SSCs remain largely unclear.

We have previously revealed that hsa-miR-1908 promotes the proliferation of human SSCs and inhibits their apoptosis by targeting KLF2.[9] As a target gene of hsa-miR-1908, it remains to be determined about the role and molecular mechanism of KLF2 in mediating the proliferation and apoptosis of human SSCs. Therefore, this study was designed to explore expression, the function, and target of KLF2 in regulating the fate decisions of human SSCs. The association between the abnormality of KLF2 and male infertility was further evaluated. This study could provide a new genetic regulatory mechanism underlying human spermatogenesis, and significantly, it might offer new targets for gene therapy for male infertility.

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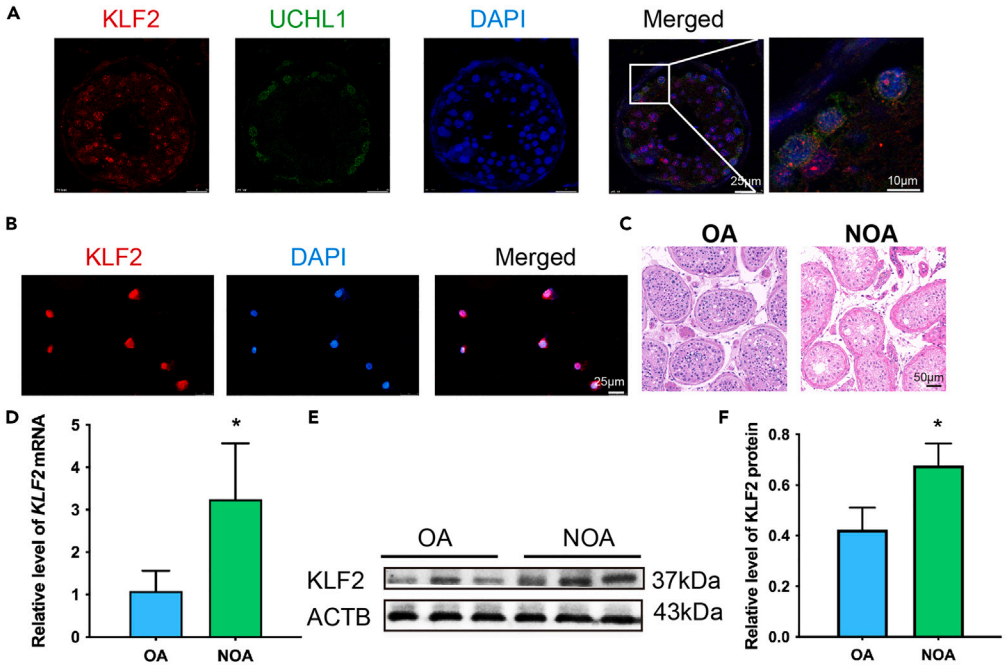

Figure 1. Cellular location and expression levels of KLF2 in human testes of OA and NOA patients

(A) Immunohistochemistry by double immunostaining showed cellular localization of KLF2 and UCHL1 in normal human testes. UCHL1 was used as a marker for human SSCs.

(B) Immunocytochemistry illustrated the expression of KLF2 in primary human SSCs, namely GFRA1-positive spermatogonia.

(C) H&E staining revealed morphological differences between OA and NOA patients. (D–F) Real-time PCR and Western blots demonstrated the relative mRNA and protein levels of KLF2 in OA and NOA testicular tissues, respectively. The relative levels of KLF2 mRNA and protein were quantified with ACTB as a loading control. * indicated statistically significant differences ($p < 0.05$) between OA and NOA patients. Data were represented as mean ± SEM.

RESULTS

KLF2 is expressed in human SSCs, and there are differences in expression levels of KLF2 in testicular tissues between non-obstructive azoospermia (NOA) and obstructive azoospermia (OA) patients

The cellular localization of KLF2 in human testes was examined by immunohistochemistry. We observed that KLF2 was expressed in a variety type of male germ cells, including spermatogonia, spermatocytes, and spermatids, in normal human testicular tissues (Figure 1A; Table S1). Notably, KLF2 was seen to be co-localized with UCHL1, a hallmark for human SSCs (Figure 1A). We isolated male germ cells from human testicular tissues by two-step enzymatic digestion and differential plating, and we sorted GFRA1^{+} spermatogonia, the putative human SSCs, by MACS. Immunocytochemistry showed that KLF2 was expressed in the nuclei of primary human SSCs (Figure 1B). H&E staining revealed normal and abnormal spermatogenesis in the seminiferous tubules of OA and NOA patients (Figure 1C). To compare gene and protein levels of KLF2 in testicular tissues between OA and NOA patients, we performed real-time PCR and Western blots and found that transcription and protein levels of KLF2 were significantly higher in NOA patients than in OA patients (Figures 1D–1F). Collectively, these data implicate that KLF2 is expressed in human SSCs, and there are differences in expression levels of KLF2 in testicular tissues between NOA patients and OA patients, indicating that KLF2 may regulate the fate decisions of human SSCs, and its abnormality is related to the pathogenesis of NOA.

KLF2 gene mutations are associated with NOA

To further determine whether KLF2 mutations are associated with the pathogenesis of NOA, whole exome sequencing (WES) and bioinformatics analysis on 777 NOA patients were performed, and we screened eight pathogenic *KLF2* variant sites located at exons (Table S2). Among them, five were missense variants (Table S2). One variant was at the variable clipping region (c.893-6C>T), and c.672C>G and c.725T>C variants were not detected in the normal control population of ESP database, 1000 Genomes database, gnomAD database, and dbSNP database, reflecting that these five variants are new variants. Notably, c.672C>G and c.725T>C were missense variants and potentially pathogenic (Table S2). Using normal population data as a control, we found that rs3745319 (gnomAD control) and rs764416555 (ExAC control) sites were more frequent than normal population. We further revealed that the frequencies of the above two genotypes and alleles in the NOA patients were statistically significant compared with the normal control group, and the OR values were all greater than 5.0, indicating that the variation of these loci was positively correlated with the risk of NOA (Table S3). Considering NOA pathogenicity prediction of *KLF2* variant sites as well as the mutation frequency and NOA risk analysis, two new gene variants were screened. Five missense variant predictions

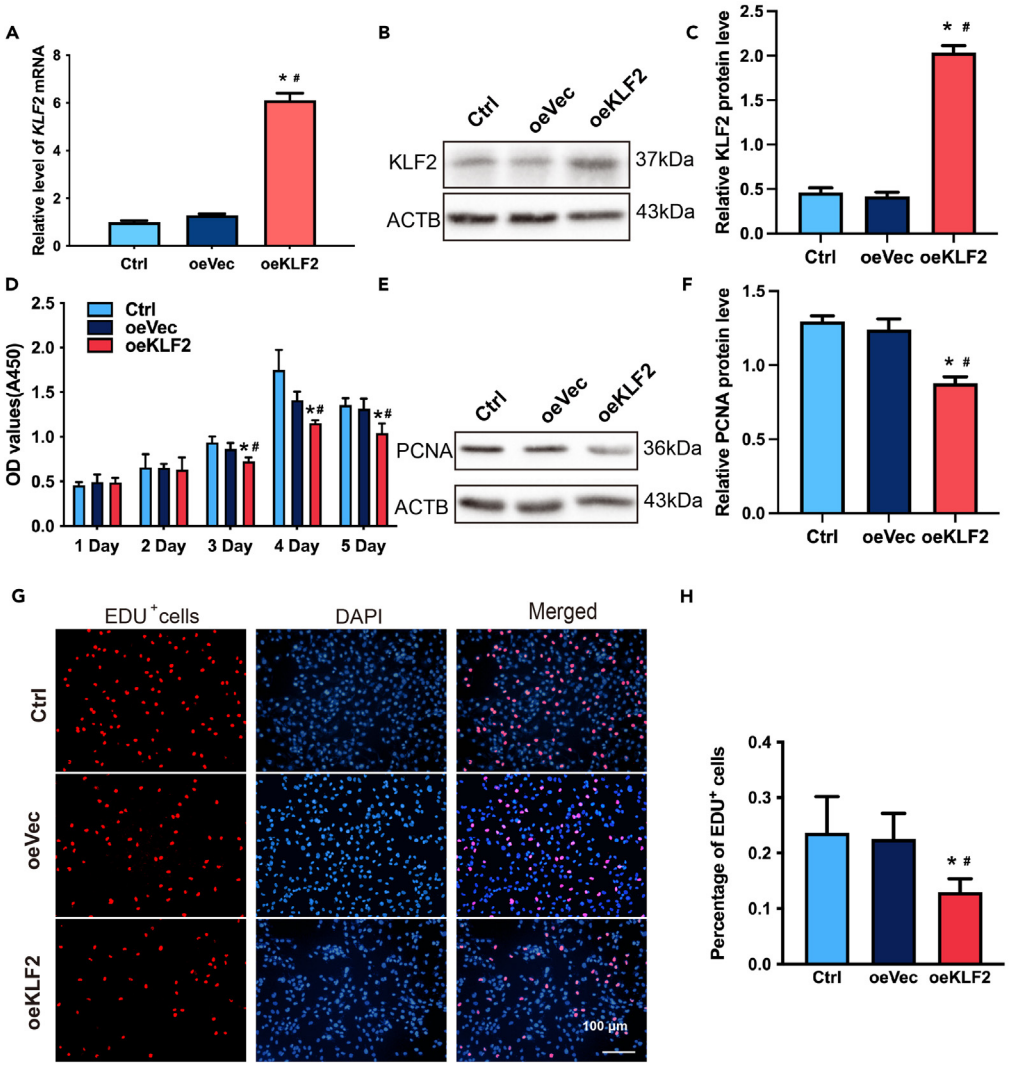

Figure 2. KLF2 overexpression suppresses the proliferation and DNA synthesis of human SSC line *in vitro*

(A–C) Real-time PCR and Western blots illustrated the relative mRNA and protein levels of KLF2 in human SSC line after transfection of *KLF2* lentiviral vectors compared with the lentiviral vector control. The relative levels of KLF2 mRNA and protein were quantified with ACTB as a loading control.

(D) CCK-8 assays displayed the growth curve of human SSC line treated with overexpression of KLF2 compared with the lentiviral vectors control for five days (E, F) Western blots demonstrated the relative protein level of PCNA in human SSC line affected by KLF2 overexpression. The band densities of PCNA protein were quantified with ACTB as a loading control.

(G and H) EDU incorporation assays showed the percentages of EDU-positive cells in human SSC line treated with lentiviral vector control compared with the KLF2-lentiviral vector. * denoted statistically significant differences ($p < 0.05$) in the KLF2-lentiviral vector compared with lentiviral vector control, whereas # indicated statistically significant differences in the KLF2-lentiviral vector ($p < 0.05$) compared with blank control. Data were represented as mean ± SEM.

might be harmful, and ACMG was classified as VUS (Table S2). Two variants were positively correlated with NOA risk. Taken together, these results suggest that *KLF2* gene variants are associated with NOA and that they may be used as potential risk predictors and targets of gene therapy for NOA.

KLF2 overexpression suppresses the proliferation and DNA synthesis of human SSC line *in vitro*

We constructed the lentivirus (pGC-FU-KLF2) for KLF2 to overexpress KLF2. Real-time PCR revealed that pGC-FU-KLF2 transfection could increase *KLF2* gene level in human SSC line (Figure 2A), and Western blots showed that pGC-FU-KLF2 enhanced KLF2 protein level in human SSC line (Figures 2B and 2C), reflecting that KLF2 could be overexpressed by pGC-FU-KLF2 in human SSC line. CCK-8 analysis demonstrated a significant decrease in proliferative activity of human SSC line by pGC-FU-KLF2 (Figure 2D), whereas Western blots exhibited a lower expression level of PCNA protein in human SSC line by pGC-FU-KLF2 compared with the vector control and lentiviral blank control (Figures 2E and 2F). Using the EDU analysis, we observed the reduction in the percentage of EDU-positive cells in human SSC line by pGC-FU-KLF2

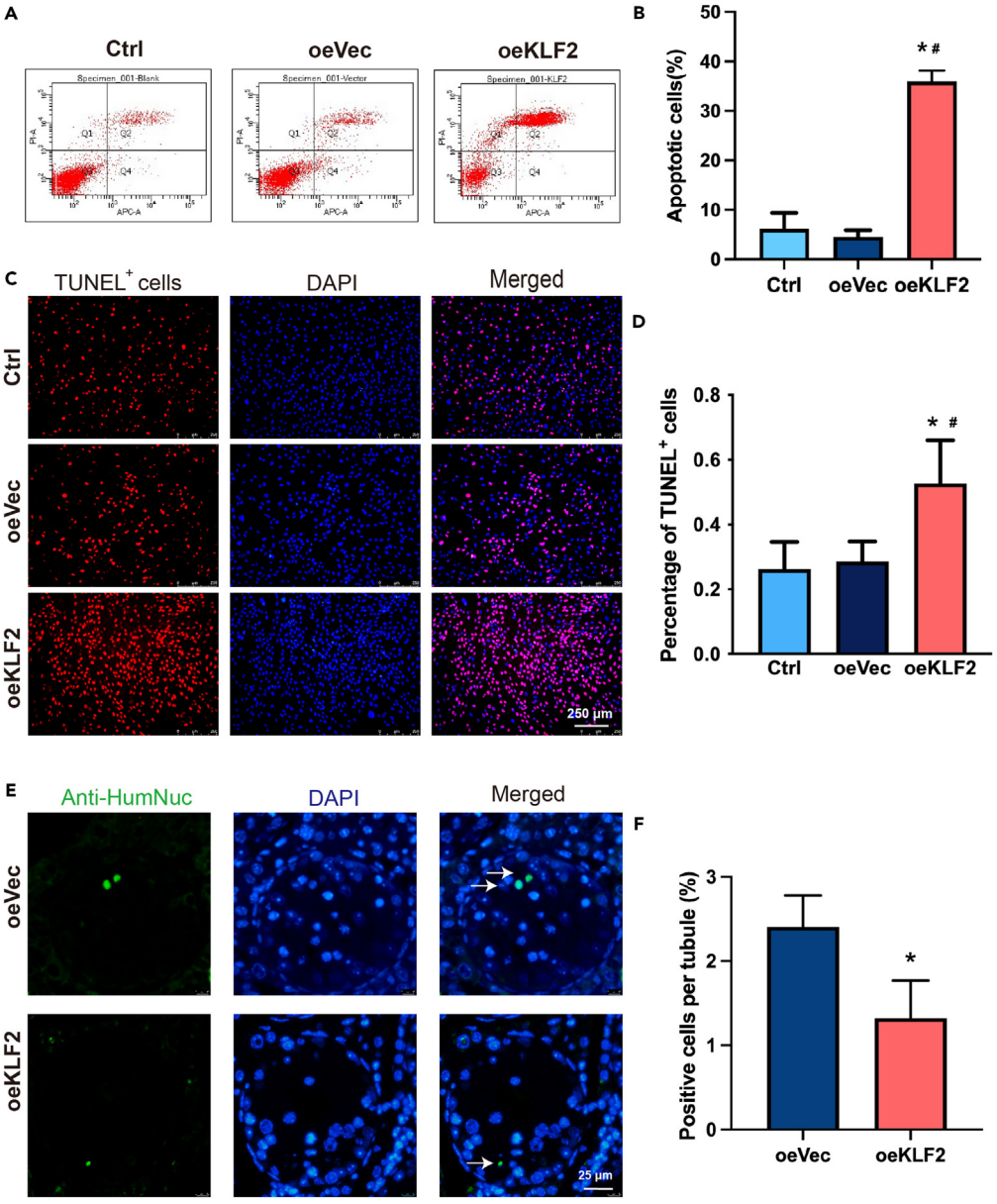

Figure 3. The impact of KLF2 overexpression on the apoptosis *in vitro* and colonization *in vivo* in the recipient mice with transplantation of human SSCs

(A and B) APC Annexin V and propidium iodide (PI) staining and flow cytometric analysis revealed the percentages of apoptosis of human SSC line affected by blank control, lentiviral vector control, and KLF2-lentiviral vector.

(C and D) TUNEL assays displayed the percentages of TUNEL-positive cells in human SSCs treated with lentiviral vector control compared with the KLF2-lentiviral vector.

(E and F) Immunohistochemistry showed the percentage of HumNuc-positive cells per seminiferous tubule in recipient mice with transplantation of human SSC line transfected with lentiviral vector control or the KLF2-lentiviral vector. * indicated statistically significant differences ($p < 0.05$) in the KLF2-lentiviral vector compared with vector control, and # implicated statistically significant differences ($p < 0.05$) in the KLF2-lentiviral vector compared with blank control. Data were represented as mean ± SEM.

(Figures 2G and 2H). Considered together, these data imply that KLF2 overexpression suppresses the proliferation and DNA synthesis of human SSCs.

KLF2 overexpression enhances the apoptosis of human SSCs *in vitro* and reduces their colonization *in vivo*

To investigate the effect of KLF2 on the apoptosis of human SSCs, flow cytometry analysis (Figures 3A and 3B) and TUNEL analysis (Figures 3C and 3D) were performed, and significant increases in apoptotic percentages were seen in human SSC line by pGC-FU-KLF2. Next, we

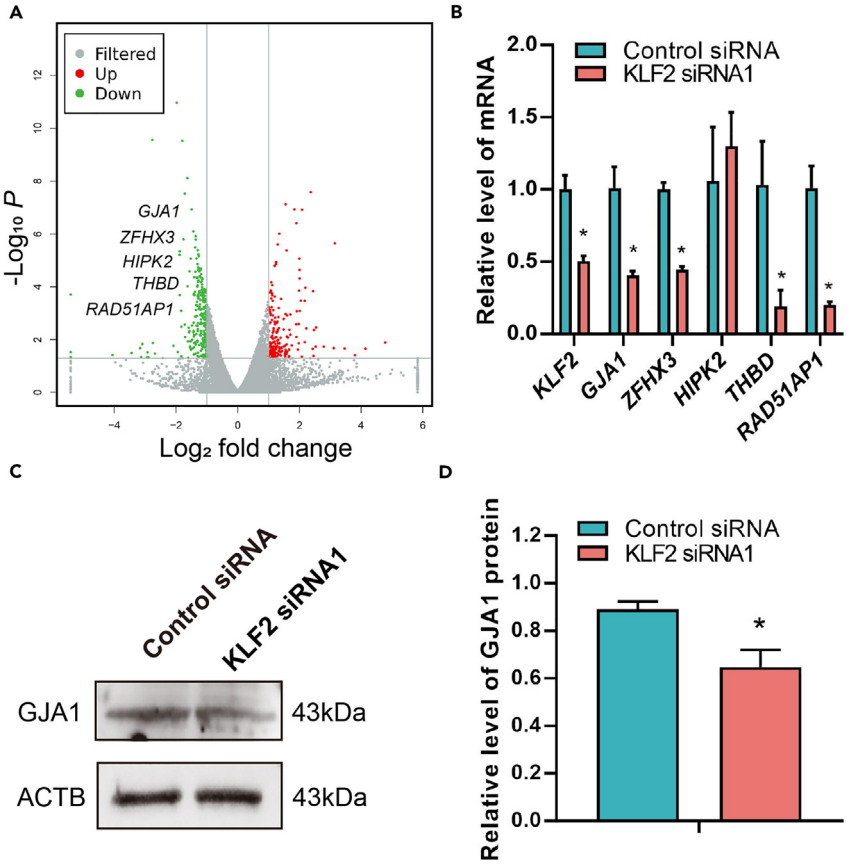

Figure 4. Identification of GJA1 as a target for KLF2 in human SSCs

(A) RNA-seq illustrated the differentially expressed genes (DEGs) between KFL2 siRNA1 and control siRNA. Red dots and blue dots represented the upregulated and downregulated genes, respectively.

(B) Real-time PCR demonstrated the relative levels of *RAD51AP1*, *THBD*, *ZFHX3*, and *HIPK2* mRNA in human SSC line after transfection of control siRNA and KFL2 siRNA1 for 48 h. The level of *GJA1* mRNA was quantified with *ACTB* as a loading control.

(C and D) Western blots showed the relative protein levels of GJA1 in human SSC line affected by KFL2 siRNA1 and control siRNA for 48 h. The band densities of GJA1 protein were quantified with ACTB as a loading control. * indicated the statistically significant differences ($p < 0.05$) between KFL2 siRNA1 and control siRNA. Data were represented as mean ± SEM.

explored the role of KLF2 in colonization of human SSCs *in vivo* using xenotransplantation. Thirty days after intraperitoneal injection of busulfan, there were no male germ cells remaining in the seminiferous tubules of recipient mice, and human SSC line with transfection of pGC-FU-KLF2 was transplanted into seminiferous tubules of recipient mice (Figure S1). The localization of human SSC line in recipient mice was detected by immunohistochemistry, showing the stable colonization of human SSC line in these mice (Figure 3E). Compared with the vector control, the proportion of HumNuc-positive cells per seminiferous tubule was decreased by pGC-FU-KLF2 (Figures 3E and 3F). Collectively, these results implicate that KLF2 overexpression accelerates the apoptosis of human SSCs *in vitro* and reduces colonization *in vivo* of human SSCs.

GJA1 is a downstream target of KLF2, and KLF2 can bind to the GJA1 promoter region in human SSCs

We have previously reported that KLF2 siRNA1 can effectively decrease gene and protein levels of KLF2 in human SSC line.[9] Here, we used RNA sequencing to screen downstream targets of the KLF2 (Figure 4A), and we found that 194 genes were upregulated and 263 genes downregulated by KLF2 siRNA1 in human SSC line compared with the control siRNA (Figures S2A and S2B). GO and KEGG analysis of the differentially expressed genes (DEGs) of RNA-seq data in human SSC line (Figures S2C and S2D) showed that DEG functions and pathways were mainly enriched in cell signal transduction. Based upon the levels of differences in gene expression and function, we identified the possible targets of KLF2 as *GJA1*, *RAD51AP1*, *THBD*, *ZFHX3*, and *HIPK2*. Real-time PCR and Western blots further demonstrated that KLF2 siRNA1 reduced GJA1 transcripts and protein levels in human SSC line compared with control siRNA (Figures 4B–4D), reflecting that GJA1 is a potential target for KLF2 in human SSCs. We utilized three-dimensional homologous model of GJA1 (Figure S3A) and found potential functional partners of GJA1, including SRC, CDH2, CTNNB1, GJP1, PKP2, DBN1, SCN5A, NEDD4, UBQLN4, EPS15, and POU5F1 (Figure S3B).

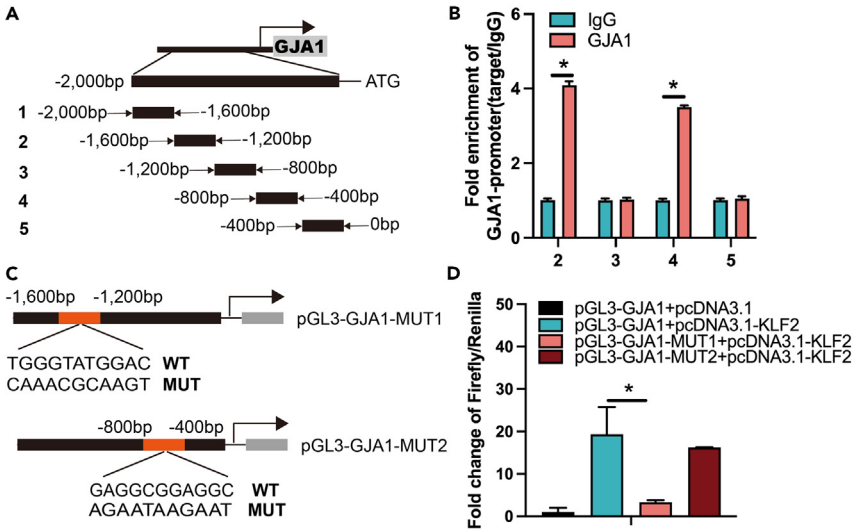

Figure 5. GJA1 is a direct downstream target of KLF2 in human SSCs

(A) A schematic diagram illustrated *GJA1* promoter region primer design strategy.

(B) ChIP-qPCR showed the differences in mRNA expression in different regions of the *GJA1* promoter region after enrichment of KLF2 protein. The levels of *GJA1* promoter mRNA were quantified with *IgG* as a loading control.

(C) A schematic diagram demonstrated KLF2 binding site sequence and mutation sequence in the *GJA1* promoter region.

(D) Dual luciferase reporter assays displayed the ratio of Firefly and Renilla between pGL3-GJA1+pcDNA3.1-KLF2 and pGL3-GJA1-MUT+pcDNA3.1-KLF2 in human SSC line. The level of ratio was quantified with pGL3-GJA1+pcDNA3.1 as a loading control. * indicated the statistically significant differences ($p < 0.05$) compared with control. Data were represented as mean ± SEM.

We analyzed GJA1 promoter sequences using JASPAR database to predict transcription factor binding sites, and our results showed that there were multiple KLF2 binding sites in the GJA1 promoter region (Table S5), suggesting that KLF2 is able to bind to the GJA1 promoter region. Furthermore, we designed primers in terms of the upstream 2,000 bp region of the GJA1 ORF region, the promoter region sequence (five pairs every 400 bp to encompass all promoter region sequences) to illustrate the binding region of transcription factor KLF2 to the GJA1 promoter region (Figure 5A). We found that KLF2 binding sites existed in the regions corresponding to the 2nd and 4th pairs of primers in the GJA1 promoter region, covered −1,600 to −1,200 bp and −800 to −400 bp regions (Figure 5B). Furthermore, based upon our results of ChIP, we performed diluciferase reporter gene validation by mutating the binding site of the GJA1 promoter region KLF2 to further clarify the exact site of KLF2 binding to the GJA1 promoter region (Figure 5C). Interestingly, we revealed that KLF2 significantly enhanced the promoter activity of GJA1 compared with pGL3-GJA1+pcDNA3.1 (Figure 5D). When the KLF2 binding site in the GJA1 promoter region was mutated, the effect of KLF2 on enhancing the activity of the GJA1 promoter was significantly weakened (Figure 5D). Considered together, these results indicate that KLF2 binds to the GJA1 promoter region −1412 to −1402 bp in human SSC line.

GJA1 silencing increases the proliferation and diminishes the apoptosis of human SSCs

To further investigate the influence of GJA1 silencing on the proliferation, DNA synthesis, and apoptosis of human SSCs, we transfected GJA1 siRNAs to silence GJA1 expression. Real-time PCR revealed that GJA1 siRNA1-3 could decrease the transcript of *GJA1* gene in human SSC line (Figure 6A), and Western blots showed that GJA1 siRNA3 reduced GJA1 protein levels in human SSC line (Figures 6B and 6C). These results indicate that GJA1 siRNA3 could knock down the GJA1 mRNA and protein expression of human SSC line. At 72 h after GJA1 siRNA3 transfection, the expression level of PCNA (proliferating cell nuclear antigen), a marker for DNA synthesis of cells, was increased in human SSC line (Figures 6D and 6E). CCK-8 analysis showed a significant increase in proliferative activity of human SSC line by GJA1 siRNA3 compared with the control siRNA (Figure 6F). Moreover, EDU assays showed that, compared with the control siRNA, the percentages of EDU-positive cells were enhanced by GJA1 siRNA3 in human SSC line (Figures 6G and 6H). The aforementioned results indicate that GJA1 silencing enhances DNA synthesis and proliferation of human SSC line. On the other hand, to determine the effect of GJA1 on apoptosis of human SSC line, TUNEL assays (Figures 6I and 6J) and flow cytometric analysis (Figures 6K and 6L) revealed that the apoptosis was decreased by KLF2 siRNA3 in human SSCs line. These results suggest that GJA1 silencing reduces the apoptosis of human SSCs.

KLF2 overexpressing rescues the increases in the proliferation, DNA synthesis, and colonization of human SSCs and the decrease in their apoptosis caused by GJA1 silencing

We have demonstrated that GJA1 is a target gene of KLF2, and it was involved in the fate decisions of human SSCs. We hypothesized that overexpression of KLF2 could rescue the effect of GJA1 silencing on human SSC line. CCK-8 assays, Western blots, and EDU assays showed

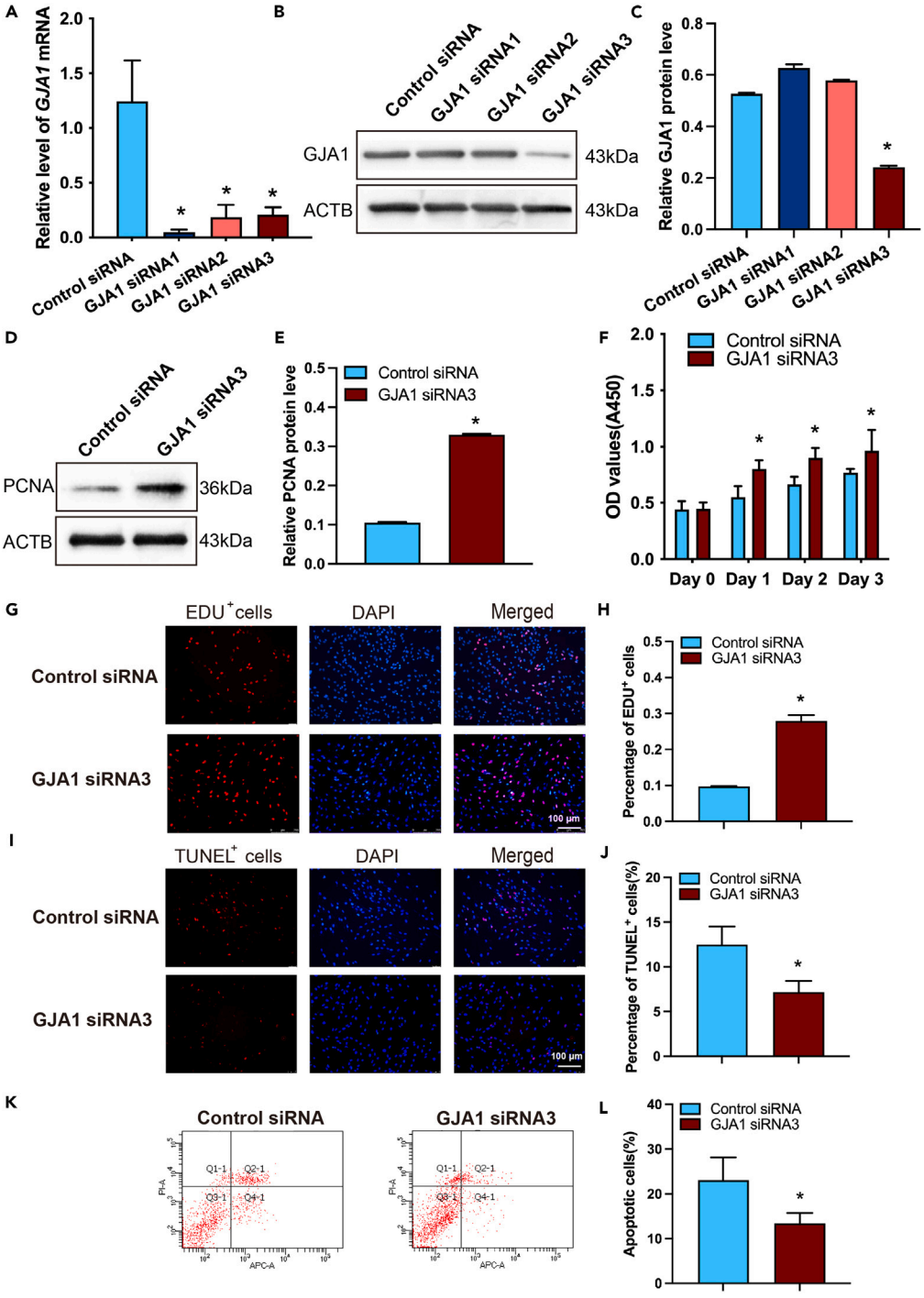

Figure 6. Influence of GJA1 silencing on the proliferation, DNA synthesis, and apoptosis of human SSCs

(A) Real-time PCR demonstrated the relative levels of *GJA1* mRNA in human SSC line after transfection of GJA1 siRNA1-3 compared with the siRNA control for 48 h. The level of *GJA1* mRNA was quantified with *ACTB* as a loading control.

(B and C) Western blots showed the relative protein levels of GJA1 in human SSC line affected by GJA1 siRNA1-3 compared with the siRNA control for 72 h. The band densities of GJA1 protein were quantified with ACTB as a loading control.

(D and E) Western blots revealed the relative protein levels of PCNA in human SSC line affected by GJA1 siRNA3 compared with the siRNA control for 72 h. The band densities of PCNA protein were quantified with ACTB as a loading control.

Figure 6. *Continued*

(F) CCK-8 assays showed the proliferation of human SSC line treated with GJA1 siRNA3 compared with the siRNA control for three days. (G,H) EDU incorporation assays revealed DNA synthesis of human SSC line treated with GJA1 siRNA3 compared with the siRNA control for 48 h.

(I–L) The percentages of apoptosis in human SSC line affected by GJA1 siRNA3 or siRNA control for 48 h as determined by flow cytometric analysis (I, J) and TUNEL assays (K, L). * indicated the statistically significant differences ($p < 0.05$) between GJA1 siRNAs and control siRNA. Data were represented as mean ± SEM.

that pGC-FU-KLF2 eliminated the increases in cell proliferation (Figure 7A), PCNA levels (Figures 7B and 7C), and EDU-positive cells (Figures 7D and 7E) of human SSC line caused by GJA1 siRNA3.

In addition, pGC-FU-KLF2 reversed a significant decrease in early and late apoptosis of human SSC line caused by GJA1 silencing (Figures 8A and 8B) as well as the decrease in TUNEL-positive cells (Figures 8C and 8D). Furthermore, the enhancement of colonization in human SSC line caused by GJA1 silencing *in vivo* was reduced by overexpression of KLF2 (Figures 8E and 8F). Taken together, these results suggest that overexpression of KLF2 can rescue the increase in colonization and decrease in the apoptosis of human SSCs caused by GJA1 knockdown.

DISCUSSION

Human SSCs have important applications in reproductive medicine and regenerative medicine because they can be induced to produce functional spermatids, reprogrammed to become embryonic stem-like cells, and transdifferentiate into functional cells of other cell lineages.[10,11] The fate decisions of human SSCs are preciously regulated by genetic and epigenetic factors.[12] In recent years, single-cell RNA sequencing has been used to reveal key genes and cell signaling pathways of human SSCs.[13,14] Nevertheless, the roles and mechanisms of these genes in fate determinations of human SSCs remain to be elucidated. We compared the large-scale differential expression profiles of miRNA in human spermatogonia and pachytene spermatocytes and found that the expression of hsa-miR-1908 in human spermatogonia was significantly higher than that in pachytene spermatocytes.[15] We have previously shown that hsa-miR-1908 promotes proliferation and inhibits apoptosis of human SSCs by targeting KLF2.[9] In this study, we have demonstrated, for the first time, that KLF2 inhibits proliferation and enhances the apoptosis of human SSCs by targeting GJA1.

To clarify the influence of KLF on the fate determinations of human SSCs, we first revealed whether KLF2 was expressed in human testes, especially in human SSCs. Previous study indicates that KLF2 belongs to the Sp1/Kruppel-like transcription factor (Sp1/KLFs) family, and it is mainly expressed in human lung tissue.[16] It has been shown that KLF2 inhibits the proliferation of various cells and enhances apoptosis through a variety of mechanisms. KLF2 is poorly expressed in tissues and cell lines, e.g., lung cancer,[17] liver cancer[18] and colorectal cancer,[19] and it suppresses cancer cell proliferation and increases the apoptosis. In addition, KLF2 plays an important role in maintaining stemness of bone marrow mesenchymal stem cells,[20] human bone marrow stromal cells,[21] and embryonic stem cells.[22] KLF2 regulates stem cell differentiation by inducing mitochondrial phagocytosis and altering mitochondrial metabolism.[23] Intestinal stromal cells mediate the proliferation and differentiation of intestinal stem cells by mediating the oxygen (ROS)-MAP3K2-ERK5-KLF2 axis.[24] Consistent with the aforementioned studies, we found, using lentivirus to construct a stable human SSCs line overexpressing KLF2, that KLF2 overexpression inhibits the proliferation of human SSC line and enhances their apoptosis. Besides proliferation and apoptosis, the colonization of human SSCs *in vivo* is of great clinical significance. For children with malignant tumors, transplantation of human SSCs has become a critical option for preservation of male fertility.[25] Laboratory animals as recipients for human SSC xenotransplantation is an alternative to examine the function of human SSCs. A variety of animal models have been found to be available as recipients for human SSC xenotransplantation, and human SSCs are immune tolerant in the testes of immune-competent mice.[26] Given the immunosuppressive effect of busulfan on immunocompetent recipient mice, we chose BALB/c mice with smaller individual differences and better overall quality as recipient animals. Thirty days after intraperitoneal injection of busulfan to recipient mice, the testes of mice assumed the depletion of male germ cells. We detected human SSCs at 30 days after xenotransplantation of human SSC line with overexpression of KLF2 into the seminiferous tubules of recipient testes via the efferent ducts, and we found that stable overexpression of KLF2 reduced the colonization ability of human SSCs in the recipient mice. Taken together, these data implicate that KLF2 overexpression leads to the inhibition of proliferation and the enhancement of apoptosis of human SSCs.

To unveil the downstream targets of KLF2 biological functions of human SSCs, we focused on GJA1 after screening by RNA sequencing, and we demonstrated that KLF2 could bind to the GJA1 promoter region and predicted the binding site in the GJA1 promoter region of KLF2 by JASPAR. Next, we used ChIP and diluciferase reporter techniques to verify the binding of KLF2 to the −1412 to −1402 bp sites in the GJA1 promoter region. In addition, we observed that KLF2 silencing decreased the expression level of GJA1 in human SSC line. GJA1 has been found to form an integral component of the Sertoli cell (SC)-SC junctional complexes that represent the anatomical base of the barrier and are involved in human spermatogenic impairment.[27] We found that GJA1 silencing stimulated cell proliferation and DNA synthesis of human SSC line and inhibited their apoptosis, which was consistent with our finding showing the effect of KLF2 knockdown on human SSC fate decisions.[9] Notably, the abovementioned enhancements in proliferation *in vitro* and colonization capacity of human SSC line *in vivo* as well as a decrease in apoptosis of these cells induced by GJA1 silencing could be attenuated by overexpression of KLF2. Therefore, these data imply that GJA1 is a target of KLF2 to mediate the fate decisions of human SSCs.

Interestingly, we found that KLF2 was expressed in human testicular tissues, and the level of KLF2 in testes of NOA patients was higher than OA patients. Based upon the matching analysis of multiple databases, we screened five new gene variants by WES in NOA patients, and notably, five missense variant predictions may be harmful, whereas ACMG was classified as VUS. Six variants were positively correlated

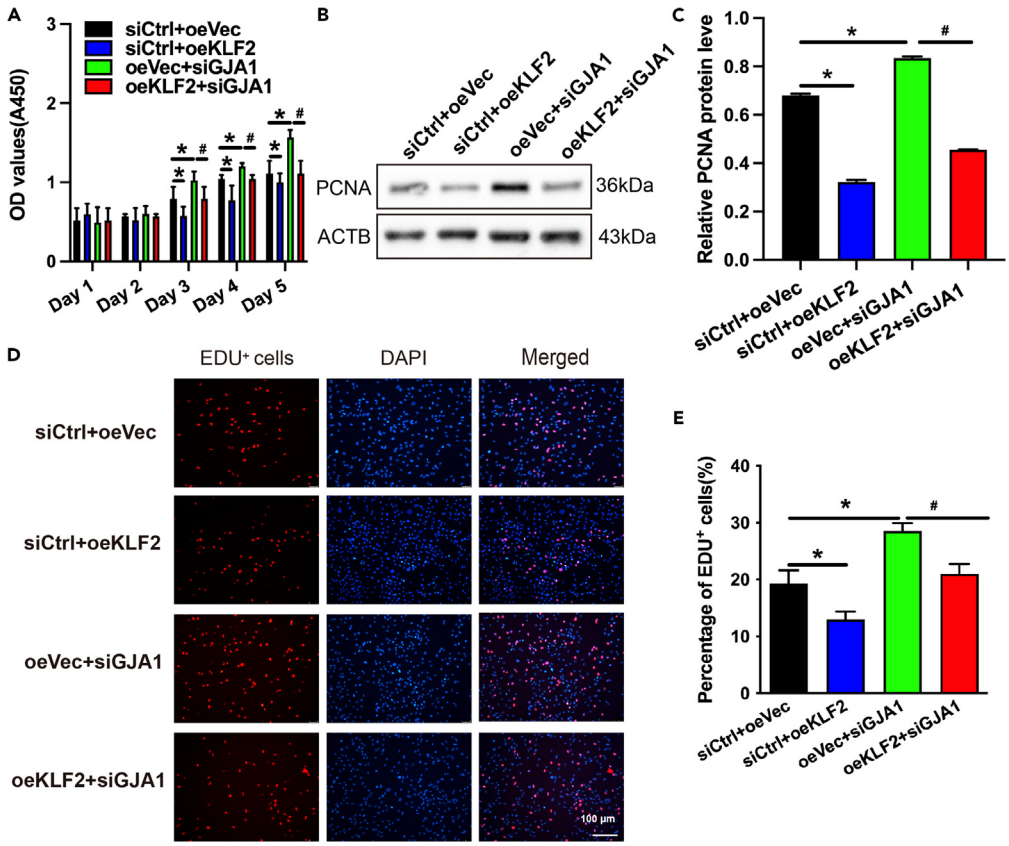

Figure 7. The function of KLF2 overexpression in controlling the effect of GJA1 silencing on the proliferation and DNA synthesis of human SSCs

(A) CCK-8 assay demonstrated the proliferation of human SSC line with overexpression of KLF2 or LV-vector and then treated with siRNA control or GJA1 siRNA3 for five days.

(B and C) Western blots showed the relative protein level of PCNA in human SSC line with overexpression of KLF2 or lentiviral vector and affected by siRNA control or GJA1 siRNA3 for 72 h. The band densities of PCNA protein were quantified with ACTB as a loading control.

(D and E) EDU assays showed the DNA synthesis of human SSC line that overexpressed KLF2 or with lentiviral vector and then treated with siRNA control or GJA1 siRNA3 for 48 h * indicated the statistically significant differences ($p < 0.05$) compared with siCtrl+oeVec group, and # denoted the statistically significant differences ($p < 0.05$) between oeKLF2+siGJA1 and oeVec+siGJA1. Data were represented as mean ± SEM.

with NOA risk. As such, higher levels of KLF2 expression and *KLF2* variation are correlated with NOA and may be used as a potential risk predictor and clinical treatment target for NOA.

In summary, we are the first to demonstrate the expression, function, and target of KLF2 in human SSCs; our data implicate that KLF2 affects the proliferation, apoptosis, and colonization ability of human SSCs via mediating GJA1. We have also revealed that abnormalities in higher levels of KLF2 expression and *KLF2* variation might be correlated with NOA. Our studies offer a new epigenetic and genetic regulatory network, namely hsa-miR-1908/KLF2/GJA1, in mediating the fate determinations of human SSCs, which is of great significance for understanding of human spermatogenesis and the treatment of male infertility.

Limitation of the study

Our data imply that KLF2 determines the fate decisions of human SSCs by interacting with GJA1. However, one limitation of this study was the absence of KLF2-knockout or KLF2-overexpression mice to further demonstrate the function and mechanism of KLF2 in regulating SSCs *in vivo*.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY

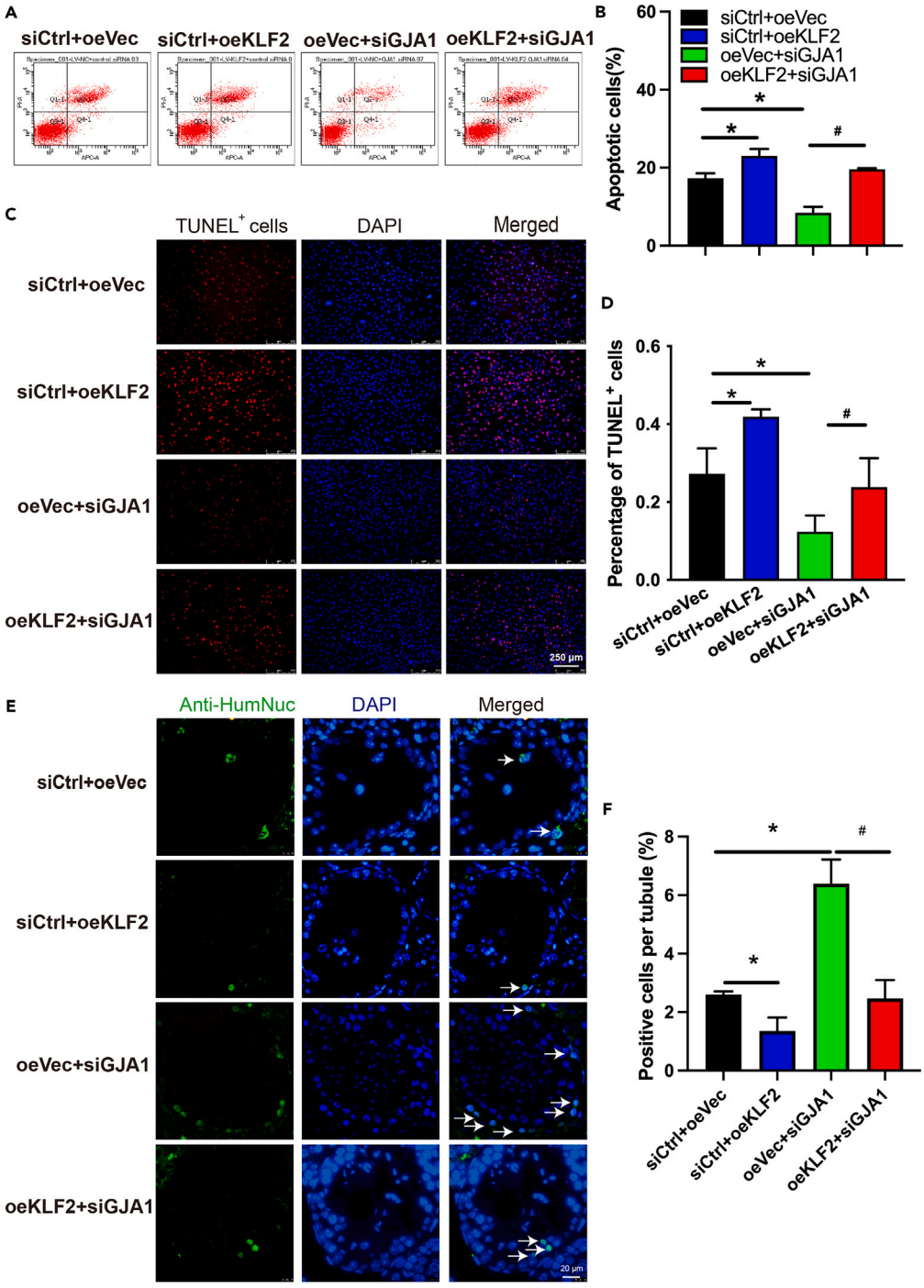

Figure 8. The role of KLF2 overexpression in mediating the influence of GJA1 silencing on the apoptosis of human SSC line *in vitro* and colonization *in vivo* in the recipient mice

(A–D) The percentages of apoptosis in human SSC line overexpressed KLF2 or lentiviral vector and affected by siRNA control or GJA1 siRNA3 for 72 h as determined by flow cytometric analysis (A, B) and TUNEL assays (C, D).

(E and F) Immunohistochemistry illustrated HumNuc-positive cells per seminiferous tubule in recipient mice with xenotransplantation of human SSC line overexpressed KLF2 or lentiviral vector and transfection of siRNA control or GJA1 siRNA3.* indicated the statistically significant differences ($p < 0.05$) compared with siCtrl+oeVec group, whereas # denoted the statistically significant differences ($p < 0.05$) between oeKLF2+siGJA1 and oeVec+siGJA1. Data were represented as mean ± SEM.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109024.

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AUTHOR CONTRIBUTIONS

W.C. performed the experiments, wrote the manuscript, and helped with data analysis. Y.C., C.L., C.H., L.D., and W.L. assisted with the experiments. Z.H. was responsible for study conception, supervision of all aspects of the laboratory experiments, data analysis, writing the manuscript, and final approval of the manuscript. All authors approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing financial interest.

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REFERENCES

1. Lotti, F., and Maggi, M. (2018). Sexual dysfunction and male infertility. Nat. Rev. Urol. *15*, 287–307. https://doi.org/10.1038/nrurol.2018.20.
2. Sun, H., Gong, T.-T., Jiang, Y.-T., Zhang, S., Zhao, Y.-H., and Wu, Q.-J. (2019). Global, regional, and national prevalence and disability-adjusted life-years for infertility in 195 countries and territories, 1990-2017: results from a global burden of disease study. Aging-Us *11*, 10952–10991. https://doi.org/10.18632/aging.102497.
3. Minhas, S., Bettocchi, C., Boeri, L., Capogrosso, P., Carvalho, J., Cilesiz, N.C., Cocci, A., Corona, G., Dimitropoulos, K., Gül, M., et al. (2021). European Association of Urology Guidelines on Male Sexual and Reproductive Health: 2021 Update on Male Infertility. Eur. Urol. *80*, 603–620. https://doi.org/10.1016/j.eururo.2021.08.014.
4. Tian, Y., and Zhou, L.-Q. (2021). Evaluating the impact of COVID-19 on male reproduction. Reproduction *161*, R37–R44. https://doi.org/10.1530/rep-20-0523.
5. Okonofua, F.E., Ntoimo, L.F.C., Omonkhua, A., Ayodeji, O., Olafusi, C., Unuabonah, E., and Ohenhen, V. (2022). Causes and Risk Factors for Male Infertility: A Scoping Review of Published Studies. Int. J. Gen. Med. *15*, 5985–5997. https://doi.org/10.2147/ijgm.S363959.
6. Aitken, R.J. (2022). The changing tide of human fertility. Hum. Reprod. *37*, 629–638. https://doi.org/10.1093/humrep/deac011.
7. Krausz, C. (2011). Male infertility: Pathogenesis and clinical diagnosis. Best Pract. Res. Clin. Endocrinol. Metab. *25*, 271–285. https://doi.org/10.1016/j.beem.2010.08.006.
8. Cui, Y., Chen, W., Du, L., and He, Z. (2023). OIP5 interacts with NCK2 to mediate human spermatogonial stem cell self-renewal and

apoptosis through cell cyclins and cycle progression and its abnormality is correlated with male infertility. Research *6*, 0162. https://doi.org/10.34133/research.0162.

9. Chen, W., Cui, Y., Liu, B., Li, C., Du, L., Tang, R., Qin, L., Jiang, Y., Li, J., Yu, X., et al. (2020). Hsa-miR-1908-3p Mediates the Self-Renewal and Apoptosis of Human Spermatogonial Stem Cells via Targeting KLF2. Mol. Ther. Nucleic Acids *20*, 788–800. https://doi.org/10.1016/j.omtn.2020.04.016.
10. Chen, Z., Sun, M., Yuan, Q., Niu, M., Yao, C., Hou, J., Wang, H., Wen, L., Liu, Y., Li, Z., and He, Z. (2016). Generation of functional hepatocytes from human spermatogonial stem cells. Oncotarget *7*, 8879–8895. https://doi.org/10.18632/oncotarget.7092.
11. Chen, Z., Niu, M., Sun, M., Yuan, Q., Yao, C., Hou, J., Wang, H., Wen, L., Fu, H., Zhou, F., et al. (2017). Transdifferentiation of human male germline stem cells to hepatocytes in vivo via the transplantation under renal capsules. Oncotarget *8*, 14576–14592. https://doi.org/10.18632/oncotarget.14713.
12. Gunes, S., and Esteves, S.C. (2021). Role of genetics and epigenetics in male infertility. Andrologia *53*, e13586. https://doi.org/10.1111/and.13586.
13. Wang, M., Liu, X., Chang, G., Chen, Y., An, G., Yan, L., Gao, S., Xu, Y., Cui, Y., Dong, J., et al. (2018). Single-Cell RNA Sequencing Analysis Reveals Sequential Cell Fate Transition during Human Spermatogenesis. Cell Stem Cell *23*, 599–614.e4. https://doi.org/10.1016/j.stem.2018.08.007.
14. Tan, K., Song, H.-W., Thompson, M., Munyoki, S., Sukhwani, M., Hsieh, T.-C., Orwig, K.E., and Wilkinson, M.F. (2020). Transcriptome profiling reveals signaling conditions dictating human spermatogonia fate in vitro. Proc. Natl. Acad. Sci. USA *117*, 17832–17841. https://doi.org/10.1073/pnas.2000362117.
15. Liu, Y., Niu, M., Yao, C., Hai, Y., Yuan, Q., Liu, Y., Guo, Y., Li, Z., and He, Z. (2015). Fractionation of human spermatogenic cells using STA-PUT gravity sedimentation and their miRNA profiling. Sci. Rep. *5*, 8084. https://doi.org/10.1038/srep08084.
16. Wani, M.A., Conkright, M.D., Jeffries, S., Hughes, M.J., and Lingrel, J.B. (1999). cDNA isolation, genomic structure, regulation, and chromosomal localization of human lung Kruppel-like factor. Genomics *60*, 78–86. https://doi.org/10.1006/geno.1999.5888.
17. Jiang, W., Xu, X., Deng, S., Luo, J., Xu, H., Wang, C., Sun, T., Lei, G., Zhang, F., Yang, C., et al. (2017). Methylation of kruppel-like factor 2 (KLF2) associates with its expression and non-small cell lung cancer progression. Am. J. Transl. Res. *9*, 2024–2037.
18. Huang, M.D., Chen, W.M., Qi, F.Z., Sun, M., Xu, T.P., Ma, P., and Shu, Y.Q. (2015). Long non-coding RNA TUG1 is up-regulated in hepatocellular carcinoma and promotes cell growth and apoptosis by epigenetically silencing of KLF2. Mol. Cancer *14*, 165. https://doi.org/10.1186/s12943-015-0431-0.
19. Wang, H.-G., Cao, B., Zhang, L.-X., Song, N., Li, H., Zhao, W.-Z., Li, Y.-S., Ma, S.-M., and Yin, D.-J. (2017). KLF2 inhibits cell growth via regulating HIF-1 alpha/Notch-1 signal pathway in human colorectal cancer HCT116 cells. Oncol. Rep. *38*, 584–590. https://doi.org/10.3892/or.2017.5708.
20. Zhou, Y., Liu, C., He, J., Dong, L., Zhu, H., Zhang, B., Feng, X., Weng, W., Cheng, K., Yu, M., and Wang, H. (2020). KLF2(+) stemness maintains human mesenchymal stem cells in bone regeneration. Stem Cell. *38*, 395–409. https://doi.org/10.1002/stem.3120.
21. Wang, H., Zhou, Y., Yu, D., and Zhu, H. (2016). Klf2 contributes to the stemness and self-renewal of human bone marrow stromal cells. Cytotechnology *68*, 839–848. https://doi.org/10.1007/s10616-014-9837-6.
22. Dunn, S.-J., Li, M.A., Carbognin, E., Smith, A., and Martello, G. (2019). A common molecular logic determines embryonic stem cell self-renewal and reprogramming. Embo Journal *38*, e100003. https://doi.org/10.15252/embj.2018100003.
23. Maity, J., Deb, M., Greene, C., and Das, H. (2020). KLF2 regulates dental pulp-derived stem cell differentiation through the induction of mitophagy and altering mitochondrial metabolism. Redox Biol. *36*, 101622. https://doi.org/10.1016/j.redox.2020.101622.
24. Wu, N., Sun, H., Zhao, X., Zhang, Y., Tan, J., Qi, Y., Wang, Q., Ng, M., Liu, Z., He, L., et al. (2021). MAP3K2-regulated intestinal stromal cells define a distinct stem cell niche. Nature *592*, 606–610. https://doi.org/10.1038/s41586-021-03283-y.
25. Wang, D., Hildorf, S., Ntemou, E., Dong, L., Pors, S.E., Mamsen, L.S., Fedder, J., Hoffmann, E.R., Clasen-Linde, E., Cortes, D., et al. (2022). Characterization and Survival of Human Infant Testicular Cells After Direct Xenotransplantation. Front. Endocrinol. *13*, 853482. https://doi.org/10.3389/fendo.2022.853482.
26. Liang, D., Sun, Q., Zhu, Z., Wang, C., Ye, S., Li, Z., and Wang, Y. (2022). Xenotransplantation of Human Spermatogonia Into Various Mouse Recipient Models. Front. Cell Dev. Biol. *10*, 883314. https://doi.org/10.3389/fcell.2022.883314.
27. Giese, S., Hossain, H., Markmann, M., Chakraborty, T., Tchatalbachev, S., Guillou, F., Bergmann, M., Failing, K., Weider, K., and Brehm, R. (2012). Sertoli-cell-specific knockout of connexin 43 leads to multiple alterations in testicular gene expression in prepubertal mice. Dis. Model. Mech. *5*, 895–913. https://doi.org/10.1242/dmm.008649.
28. Hou, J., Niu, M., Liu, L., Zhu, Z., Wang, X., Sun, M., Yuan, Q., Yang, S., Zeng, W., Liu, Y., et al. (2015). Establishment and Characterization of Human Germline Stem Cell Line with Unlimited Proliferation Potentials and no Tumor Formation. Sci. Rep. *5*, 16922. https://doi.org/10.1038/srep16922.
29. He, W.-B., Tu, C.-F., Liu, Q., Meng, L.-L., Yuan, S.-M., Luo, A.-X., He, F.-S., Shen, J., Li, W., Du, J., et al. (2018). DMC1 mutation that causes human non-obstructive azoospermia and premature ovarian insufficiency identified by whole-exome sequencing. J. Med. Genet. *55*, 198–204. https://doi.org/10.1136/jmedgenet-2017-104992.
30. Zhang, W., Chen, W., Cui, Y., Wen, L., Yuan, Q., Zhou, F., Qiu, Q., Sun, M., Li, Z., and He, Z. (2021). Direct reprogramming of human Sertoli cells into male germline stem cells with the self-renewal and differentiation potentials via overexpressing DAZL. Stem Cell Rep. *16*, 2798–2812. https://doi.org/10.1016/j.stemcr.2021.09.011.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-KLF2	Abcam	Cat# ab203591; RRID: AB_3086613
Mouse anti-UCHL1	Bio-Rad	Cat# MCA475; RRID: AB_2210503
Rabbit polyclonal anti-GFRA1	Abcam	Cat# ab8026; RRID: AB_306208
Rabbit monoclonal anti-GJA1	Abcam	Cat# ab235585; RRID: AB_3086612
Mouse monoclonal anti-PCNA	Cell Signaling Technology	Cat# 2586; RRID: AB_2160343
Mouse monoclonal anti-ACTB	Proteintech	Cat# HRP-60008; RRID: AB_2819183
Mouse monoclonal anti-human nuclear antigen	Abcam	Cat# ab191181; RRID: AB_2885016
Biological samples		
Testicular tissues	N/A	From OA and NOA patients
Peripheral blood	N/A	From NOA patients
Chemicals, peptides, and recombinant proteins		
FBS	Gibco	Lot.1640960
DMEM/F-12	Gibco	Cat#21331020
Penicillin-Streptomycin	Sigma-Aldrich	Cat# P4333
Trypsin	Gibco	Cat# 25200114
Hyaluronidase	Sigma-Aldrich	Cat# H1115000
BSA	Sigma-Aldrich	Cat# 10711454001
DNase I	Gibco	Cat# 18047019
CollagenaseType IV	Gibco	Cat# 17104019
lipofectamine 3000	Life Technologies	Cat# L3000150
RNAiso Plus regent	Takara	Cat# 9109
HiScript II Q RT SuperMix	Vazyme	Cat# R223-01
Power SYBR Green PCR Master Mix	Applied Biosystems	Cat# 4367659
16% formaldehyde	Cell Signaling Technology	Cat# 12606S
Busulfan	Sigma-Aldrich	Cat# 55-98-1
RIPA	Beyotime	Cat# P0013B
PMSF	Beyotime	Cat# ST506
Critical commercial assays		
QIAamp DNA blood midi kit	Qiagen	Cat# 51183
SimpleChIP® Enzymatic Chromatin IP Kit	Cell Signaling Technology	Cat# 9003
Dual-Glo® Luciferase Reporter Assay System	Promega	Cat# E2920
CCK-8 assays	Dojin Laboratories	Cat# CK04
Cell-Light EdU Apollo567 In Vitro Kit	Ribobio	Cat# C10310
TUNEL assay	Servicebio	Cat# G1502
Experimental models: Cell lines and cells		
Cell lines: Human SSC line	Hou, 2015, ref. [11]	N/A
Cells: Human primary SSCs	N/A	From OA and NOA patients

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Mouse: BALB/c	Hunan SJA Laboratory Animal Co., Ltd	N/A
Oligonucleotides		
GJA1 siRNAs	This paper	N/A
Primers	This paper	N/A
Recombinant DNA		
pGL3-GJA1+pcDNA3.1	This paper	N/A
pGL3-GJA1+pcDNA3.1	This paper	N/A
pGL3-GJA1+pcDNA3.1	This paper	N/A
pGL3-GJA1+pcDNA3.1	This paper	N/A
Software and algorithms		
BD FACSDiva software	BD Biosciences	N/A
FlowJo v10	BD Biosciences	https://www.flowjo.com
Prism 8	GraphPad	https://www.graphpad.com

RESOURCE AVAILABILITY

Lead contact

Further information and request for reagents and resources should be directed to and will be fulfilled by the lead contacts, Dr. Zuping He (zupinghe@hunnu.edu.cn).

Materials availability

This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Male wild-type BALB/c mice of 8 weeks and 20–25 g were used, and they were obtained from Hunan SJA Laboratory Animal Co. Ltd, China. Testicular biopsies and peripheral blood from OA or NOA patients who underwent microdissection and testicular sperm extraction were obtained from Hunan Guangxiu Hospital affiliated to Hunan Normal University. The studies involving human participants and animal experimental procedures were approved by the Ethic Committee of Hunan Normal University (2021079). The informed consent was obtained from NOA and OA patients to participate in this study. The information on OA and NOA patients was shown in Table S1.

MATERIALS AND METHODS

Immunohistochemistry and haematoxylin and eosin (H&E) staining

Obstructive azoospermia (OA) and non-obstructive azoospermia (NOA) patients were used and classified according to WHO diagnostic criteria without karyotype abnormality and AZF microdeletion.[3] Testicular biopsies from OA or NOA patients who underwent microdissection and testicular sperm extraction were obtained from Hunan Guangxiu Hospital affiliated to Hunan Normal University. Immunohistochemistry was performed on 5 μm testis tissue sections using standard protocols. Immunostaining was conducted with antibodies against KLF2 (ab203591, 1:100, Abcam) and UCHL1 (MCA4750, 1:100, Bio-Rad). The positive cells were observed under a fluorescence microscopy or confocal microscopy (Leica, Germany). To evaluate OA and NOA patients' testicular histology, sections were stained with haematoxylin and eosin (H&E).

Isolation of human SSCs from obstructive azoospermia (OA) patients

Male germ cells were separated by two-step enzyme digestion. Testicular tissues from OA patients were digested by enzyme I (2 mg/mL type IV collagenase and 1 μg/μL DNase I) to obtain seminiferous tubules. Male germ cells were isolated by enzyme II (4 mg/mL collagenase IV, 2.5 mg/mL hyaluronidase, 2 mg/mL trypsin and 1 μg/μL DNase I) and followed by differential plating. GFRA1-positive cells, namely primary human spermatogonial stem cells (SSCs), were separated by MACS using anti-GFRA1 antibody (ab8026, 1:200, Abcam) according to the procedures of the instruction of manufacturer (Miltenyi Biotec).

Human SSC line culture and siRNA & lentiviral transfection

Human SSC line was established and cultured as we described previously.[28] Human SSC line was transfected with siRNAs, including GJA1 siRNA1, GJA1 siRNA2, GJA1 siRNA3, or control siRNA (GenePharma, China), utilizing lipofectamine 3000, and it was transfected with lentiviruses that overexpressed KLF2 vectors (Genechem, China) using lipofectamine 3000 pursuant to the instruction of manufacture (Life Technologies, USA). Puromycin selection was applied at 2 μg/mL to acquire cells with stable KLF2 overexpression. After transfection of siRNAs or lentiviruses, cells were collected at 48 or 72 h for detecting gene or protein expression and functional studies. The sequences of GJA1 siRNAs were shown in Table S4.

Real-time PCR

Total RNA was extracted from the testicular tissues or cells by the RNAiso Plus regent (Takara, Japan). Reverse transcription (RT) was conducted using the HiScript II Q RT SuperMix for qPCR (Vazyme, China). Quantitative real-time PCR reactions were performed using Power SYBR Green PCR Master Mix (Applied Biosystems, USA) and real-time PCR system (Applied Biosystems). PCR products were quantified by the comparative CT (Threshold Cycle) method. The primer sequences of genes for real-time PCR were listed in Table S6.

Western blots

After RIPA and PMSF lysis of tissues (50 mg) or cells, protein concentrations were measured using the BCA protein assay kit. Western blots were performed using standard protocols. Immunostaining was performed with antibodies against KLF2 (ab203591, 1:1000, Abcam), GJA1 (ab235585, 1:1000, Abcam), PCNA (2586, 1:2000, Cell Signaling Technology), and ACTB (60008, 1:2000, Proteintech). The intensities of protein bands were visualized using chemiluminescence (Bio-Rad, USA).

RNA-sequencing and whole-exome sequencing (WES)

RNA was isolated for RNA sequencing (Oebiotech, China). As previously described,[29] QIAamp DNA blood midi kit (Qiagen, Hilden, Germany) was employed to extract genomic DNA from peripheral blood samples of NOA patients according to the manufacturer's instruction. WES was performed using the HiSeq2000 sequencing platform (Illumina, San Diego, California, USA), and the PCR replicates were removed and sorted by excluding the adapters and aligning with the NCBI GRCh38 genome. Finally, WES data analysis was performed using the Genomic Analysis Toolkit (GATK-4.1.9.0).

Chromatin co-immunoprecipitation (ChIP)

ChIP assays were carried out in terms of the manual of manufacturer (Cell Signaling Technology). Briefly, cells were cross-linked with 1% formaldehyde (Cell Signaling Technology), and formaldehyde was inactivated by the addition of 1M glycine. Chromatin extracts containing DNA fragments with an average size of 150–900 bp were immunoprecipitated using anti-KLF2 antibody (ab203591, 1:30, Abcam) or IgG (Cell Signaling Technology). The precipitated DNA was analyzed via real-time PCR.

Luciferase reporter assay

After transfection of pGL3-GJA1+pcDNA3.1, pGL3-GJA1+pcDNA3.1-KLF2, pGL3-GJA1-MUT1+pcDNA3.1-KLF2, and pGL3-GJA1-MUT2+-pcDNA3.1-KLF2 (Genecreate, China) for 48 h, luciferase activities were measured by the Dual-Glo Luciferase Reporter Assay System (E2920, Promega) using the tube luminometer (Berthold, Germany) according to the manufacturer's protocol. Data were normalized to pGL3-GJA1+-pcDNA3.1-transfected cells.

CCK-8 assays

The cells were harvested at 0 to 5 days after KLF2 overexpression and/or transfection of GJA1 siRNAs. After 3 h of incubation with CCK-8 solution (Dojin Laboratories, Japan), the absorbance of 450 nm was determined by a microplate reader.

EDU incorporation assay

At 48 h after cell transfection, EDU immunostaining was performed with Apollo staining reaction buffer, and cell nuclei were stained with Hoechst 33342 pursuant to the instruction of manufacturer (RiboBio, China). The EDU-positive cells were counted from at least 500 cells under a fluorescence microscopy (Leica, Germany).

Flow cytometric analysis

The apoptosis of human SSCs was determined at 48 h after KLF2 overexpression and/or transfection of GJA1 siRNAs. Cells were resuspended with Annexin V Binding Buffer (Biolegend, UK), and APC Annexin V and propidium iodide (PI) solution were added to cells. After 15 min incubation at room temperature in the dark, the cells were analyzed by flow cytometry with C6 instrument (BD Biosciences, USA).

TUNEL assay

The apoptosis of human SSCs was also assessed by TUNEL assay. At 48 h after cell transfection, TUNEL immunostaining was performed with TMR-5-dUTP Labeling Mix/TdT enzyme buffer, and cell nuclei were stained with Hoechst 33342 (Servicebio, China). The TUNEL-positive cells were counted from at least 500 cells under a fluorescence microscopy (Leica, Germany).

Xenotransplantation of human SSCs into mice

Intraperitoneal injection of busulfan (40 mg/kg) (Sigma-Aldrich, USA) into male mice (BALB/c) was conducted at 8 weeks to remove their male germ cells. At 4 weeks after busulfan treatment, 10 μL (1×10^6) of human SSC line without or with KLF2 overexpression and/or transfection of GJA1 siRNA3 were transplanted into the seminiferous tubules of recipient testes via the efferent ducts.[30] At four weeks after xenotransplantation, the recipient mice were euthanized, and their testes were employed for the paraformaldehyde fixation and immunohistochemical staining for human nuclear antigen (HumNuc, ab191181, 1:100, Abcam), a protein specifically reactive with human.

Statistical analysis

Data were expressed as means ± SEM. Comparisons between two groups were performed using unpaired t-test, and p-values < 0.05 were considered statistically significant. When individual data were shown, there were representatives of at least three independent experiments.