Cellular and Molecular Life Sciences (2024) 81:391
https://doi.org/10.1007/s00018-024-05414-w

ORIGINAL ARTICLE

LncRNA ACVR2B-as1 interacts with ALDOA to regulate the self-renewal and apoptosis of human spermatogonial stem cells by controlling glycolysis activity

Zhipeng Xu[1] · Cai Lv[3] · Jun Gao[1] · Yinghong Cui[2] · Wei Liu[2,3] · Zuping He[2,5] · Leye He[1,4]

Received: 24 April 2024 / Revised: 3 August 2024 / Accepted: 14 August 2024

Abstract

Human spermatogonial stem cells (SSCs) have significant applications in reproductive medicine and regenerative medicine because of their great plasticity. Nevertheless, it remains unknown about the functions and mechanisms of long non-coding RNA (LncRNA) in regulating the fate determinations of human SSCs. Here we have demonstrated that LncRNA ACVR2B-as1 (activin A receptor type 2B antisense RNA 1) controls the self-renewal and apoptosis of human SSCs by interaction with ALDOA via glycolysis activity. LncRNA ACVR2B-as1 is highly expressed in human SSCs. LncRNA ACVR2B-as1 silencing suppresses the proliferation and DNA synthesis and enhances the apoptosis of human SSCs. Mechanistically, our ChIRP-MS and RIP assays revealed that ACVR2B-as1 interacted with ALDOA in human SSCs. High expression of ACVR2B-as1 enhanced the proliferation, DNA synthesis, and glycolysis of human SSCs but inhibited their apoptosis through up-regulation of ALDOA. Importantly, overexpression of ALDOA counteracted the effect of ACVR2B-as1 knockdown on the aforementioned biological processes. Collectively, these results indicate that ACVR2B-as1 interacts with ALDOA to control the self-renewal and apoptosis of human SSCs by enhancing glycolysis activity. This study is of great significance because it sheds a novel insight into molecular mechanisms underlying the fate decisions of human SSCs and it may offer innovative approaches to address the etiology of male infertility.

Keywords LncRNA ACVR2B-as1 · Spermatogonial stem cells · Self-renewal · Apoptosis · Glycolysis

Introduction

Infertility, characterized by the inability to conceive, has emerged as a serious concern for human reproductive health, which affects 17.5% of the populations globally [1]. It has been estimated that male factor contributes to 30%–50% of infertility cases [2]. Non-obstructive azoospermia (NOA) is the most severe manifestation of male infertility, and it constitutes around 10% of infertile male populations [3]. The isolation and transplantation of spermatogonial stem cells (SSCs) provide promise for restoring fertility, thereby enabling natural pregnancy [4]. SSCs serve as the initial cells for normal spermatogenesis, [5] which is influenced by various factors, including epigenetic and genetic factors. Notably, SSCs possess great plasticity and have significant applications in regenerative and reproductive medicine, because they can directly transdifferentiate into functional cells, including mature hepatocytes, neurons, uterine, and spermatids [6]. As such, it is of particular significance to

✉ Zuping He
zupinghe@hunnu.edu.cn

✉ Leye He
leyehe@csu.edu.cn

1 Department of Urology, The Third Xiangya Hospital, Central South University, Changsha 410011, China

2 Key Laboratory of Model Animals and Stem Cell Biology in Hunan Province, Engineering Research Center of Reproduction and Translational Medicine of Hunan Province, Hunan Normal University School of Medicine, Changsha 410013, Hunan, China

3 Department of Urology, Haikou Municipal Hospital, Haikou, Hainan, China

4 Department of Pharmacy, The Third Xiangya Hospital, Central South University, Changsha 410011, China

5 Shanghai Key Laboratory of Reproductive Medicine, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China

Published online: 10 September 2024

uncover the molecular mechanisms regulating the fate determinations of human SSCs [7].

Long non-coding RNAs (LncRNAs) have more than 200 nucleotides (200 nt) in length, [8] and they are required for regulating mammalian SSCs [9]. For instance, LncRNA033862 serves as an antisense transcript of *Gfra1*, and it controls the proliferation, DNA synthesis, and survival of mouse SSCs [10]. Furthermore, LncRNA Mrhl is involved in the differentiation of mouse SSCs via interacting with SOX8 and negative regulation of Wnt signaling [11, 12]. LncRNA AK015322 stimulates the proliferation of mouse SSCs by antagonizing miRNA-19b-3p [13]. Nevertheless, due to the differences in cell types and biochemical phenotypes between mouse and human SSCs, molecular mechanisms underlying fate decisions of mouse SSCs may not be applied to humans [14]. Therefore, it is crucial to unveil the functions of LncRNAs and their regulatory mechanisms that govern the proliferation and apoptosis of human SSCs.

It has recently been reported that glycolysis is involved in controlling the activities of SSCs [15–18]. Single-cell RNA sequencing reveals numerous genes that are related to glycolysis in SSCs [5]. *Fructose-bisphosphate aldolase A* (*ALDOA*) is localized at chromosome 16q22-q24, and notably, it is a pivotal enzyme in glycolysis [19]. ALDOA is expressed in muscle tissue, [20] gastric cancer, [21] pancreatic carcinoma, [22] liver carcinoma, [23] and glioblastoma [24]. POU2F1 has been shown to stimulate the proliferation and chemoresistance of colon cancer cells by increasing glycolysis [25]. ALDOA is significantly elevated by TGF-β, which is correlated with the proliferation and metastasis of pancreatic carcinoma cells [26]. However, the specific functions of ALDOA in controlling human SSCs and its underlying mechanisms remain elusive.

To identify the LncRNAs essential for human SSC proliferation, we conducted RNA sequencing. Significantly, we found that the expression level of LncRNA ACVR2B-as1 was up-regulated by 10% FBS in human SSCs. Subsequently, we demonstrated the crucial functions of LncRNA ACVR2B-as1 in mediating the self-renewal, apoptosis, and glycolysis of human SSCs. Our data implicate that LncRNA ACVR2B-as1 regulates the self-renewal and apoptosis of human SSCs via interaction with ALDOA to control glycolysis activity. This study is thus of particular significance because it provides a novel epigenetic regulatory mechanism controlling the fate decisions of human SSCs and offers new avenues for gene therapy of male infertility.

Results

LncRNA ACVR2B-as1 is highly upregulated in human SSCs in response to 10% FBS and it is localized at the cytoplasm of human SSCs

We initially verified the identity of the human SSC line as the human SSCs. RT-PCR analysis demonstrated the expression of *SV40* and numerous gene markers for human spermatogonia, germ cells and human SSCs, including *MAGEA4*, *VASA*, *GFRA1*, *GPR125*, *RET*, *THY1*, *UCHL1*, and *PLZF* (Figure S1A). Furthermore, immunocytochemistry (ICC) illustrated the presence of specific proteins, e.g., SV40, GPR125, UCHL1, GFRA1, THY1, and PLZF (Figure S1B-S1G) in this cell line. These data implicate that this human SSC line was human primary SSCs with molecular phenotype.

In the human SSC line, we observed that after serum starvation, the level of PCNA, a hallmark for cellular proliferation, was increased by 2.49-fold when it was cultured with 10% FBS in comparison to 0.5% FBS for 24 h (Figure S2A). To identify novel LncRNAs essential for human SSC proliferation, we employed RNA sequencing (RNA-seq) to compare the global LncRNA profiles of human SSCs between being cultured with 10% FBS and 0.5% FBS (see diagram in Figure S2B). Significantly, we identified 129 up-regulated and 112 down-regulated LncRNAs by 10% FBS in human SSC line (Fig. 1A-1B). We verified the up-regulation of five LncRNAs, and notably, we found that LncRNA ACVR2B-as1 exhibited the highest elevation (144.6 ± 43.39, $P < 0.05$) by 10% FBS in the human SSC line (Fig. 1C). To investigate potential sequence alterations of LncRNA ACVR2B-as1 in human SSCs, the transcripts were segmented into three parts (P1, P2, and P3) using the known sequence of ACVR2B-as1, and we designed primers (Table S6) and performed 5' and 3' RACE (Fig. 1D**).** Subsequent Sanger sequencing showed the full-length transcript of ACVR2B-as1 in the human SSC line (Figure S3). To elucidate the cellular localization of LncRNA ACVR2B-as1, we harvested human SSCs and separated their nucleus and cytoplasm. Subsequently, we conducted qPCR to determine the expression of LncRNA ACVR2B-as1. We revealed that $83.22 \pm 2.43\%$ of LncRNA ACVR2B-as1 was located at the cytoplasm of human SSCs (Fig. 1E). Furthermore, our FISH assay illustrated a high level of LncRNA ACVR2B-as1 transcript in the cytoplasm of human SSCs (Fig. 1F) and in the cytoplasm of human testis with normal spermatogenesis (Fig. 1G).

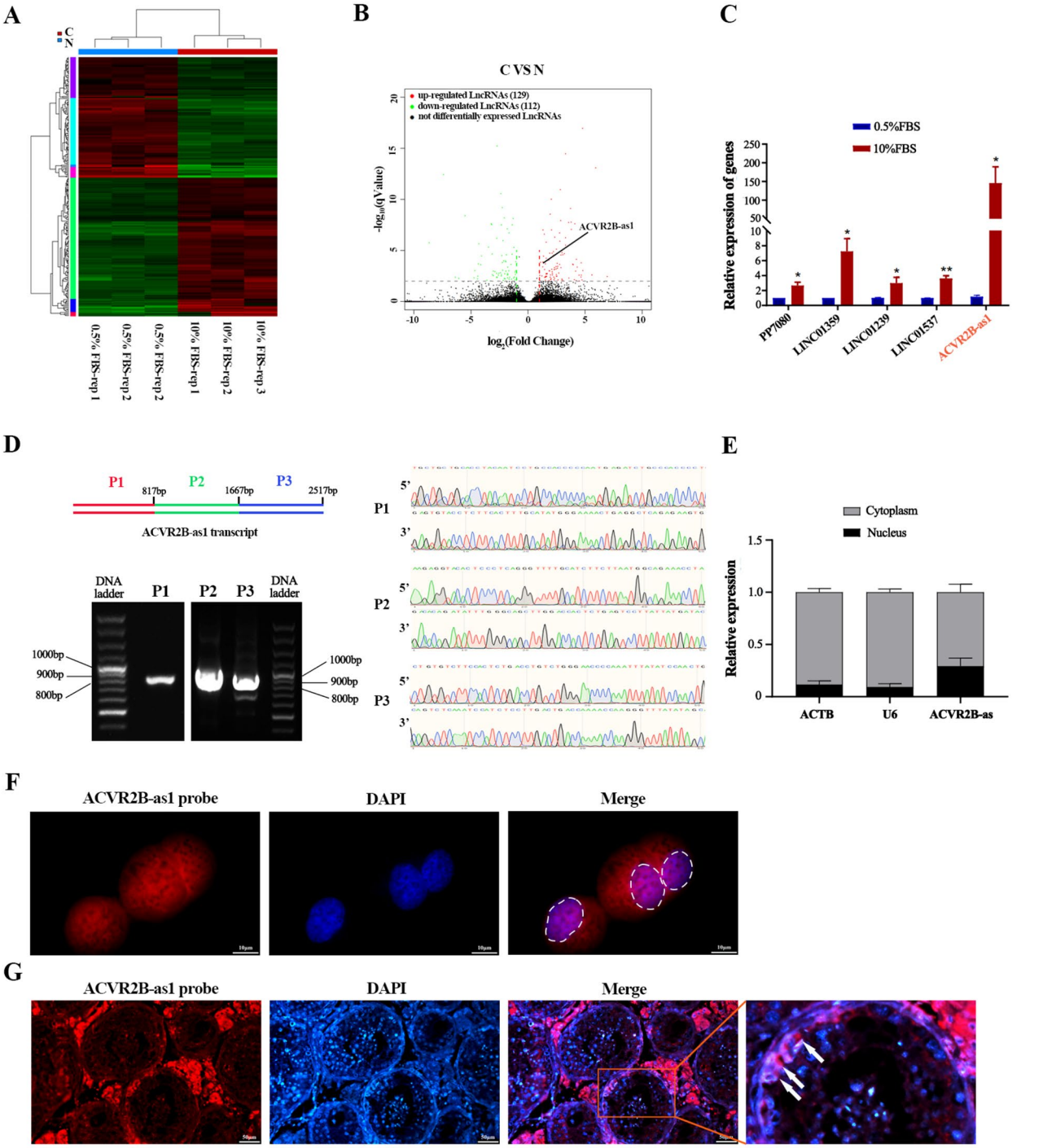

Fig. 1 LncRNA ACVR2B-as1 was elevated by 10% FBS in human SSC line and it was expressed in human SSCs. **A**, Heatmap analysis indicated the 231 differentially expressed lncRNAs in human SSC line between 10% FBS and 0.5% FBS. **B**, Volcano plot displayed the distribution of the differentially expressed lncRNAs. The differentially expressed lncRNAs were determined based upon |Fold change|≥2 and P value < 0.05. **C**, The qPCR showed the highest level of lncRNA ACVR2b-as1 in human SSC line and expression of other lncRNAs PP7080, LINC 01359, LINC 01239, LINC 01537 that exhibited expression changes in human SSC line by 10% FBS. **D**, The 5', 3'-terminal unknown sequence clone strategy of ACVR2B-as1 in human SSC line by rapid-amplification of cDNA ends (RACE). **E**, Subcellular fractionation of ACVR2B-as1 in the human SSC line by qPCR. **F-G**, Representative pictures of FISH used to detect the subcellular localization of ACVR2B-as1 in human SSC line (**F**) and human testes (**G**). ACVR2B-as1 were stained with specific probe of ACVR2B-as1 (red), while nuclei were labelled with DAPI (blue)

LncRNA ACVR2B-as1 silencing suppresses the proliferation and DNA synthesis and enhances apoptosis of the human SSCs

Our KEGG analysis indicated that the differentially expressed lncRNAs of RNA-seq were mainly enriched in cell growth and death, cell motility and others (Fig. 2A). We asked whether ACVR2B-as1 could affect the self-renewal and apoptosis of human SSCs. Thus, three siRNAs targeting LncRNA ACVR2B-as1 were employed to suppress its expression and investigate its effect on the fate determinations of human SSCs. Our qPCR analysis unveiled a notable decrease in the expression levels of ACVR2B-as1 in human SSCs upon treatment with ACVR2B-as1 siRNA1, ACVR2B-as1 siRNA2, and ACVR2B-as1 siRNA3 (Figure S4). CCK-8 assay indicated a dramatic decline in the number of human SSCs following treatment with ACVR2B-as1 siRNAs on days 4 to 5 (Fig. 2B). Moreover, PCNA expression level (Fig. 2C and D) and EDU-positive cell percentages (Fig. 2E and F) were decreased in human SSCs by treatment with LncRNA ACVR2B-as1 siRNAs. Additionally, flow cytometry (Figure S5A-S5B) and TUNEL (Figure S5C-S5D) assays demonstrated an increase in apoptotic and TUNEL-positive cells in the human SSC line following ACVR2B-as1 silencing. Considered together, these findings suggest that ACVR2B-as1 silencing leads to decreases in proliferation and DNA synthesis and enhancement of apoptosis in human SSCs.

LncRNA ACVR2B-as1 modulates ALDOA expression through binding and regulation in human SSCs

To seek the targets of LncRNA ACVR2B-as1 in human SSCs, we conducted a ChIRP-MS assay to explore the proteins that bind to ACVR2B-as1 and facilitate its impact on the fate decisions of these cells. Subsequently, pathway enrichment analysis was performed using the KEGG analysis, which implicates the significant enrichment of the interacting proteome of ACVR2B-as1 in pathways, e.g., glycolysis or gluconeogenesis (Fig. 3A). Additionally, our Western blot revealed a decrease of ALDOA expression in human SSCs upon ACVR2B-as1 silencing (Fig. 3B and C). To further explore the physical interaction between ACVR2B-as1 and ALDOA, we conducted a RIP assay using an anti-ALDOA, and we found a notable enrichment of ACVR2B-as1 by anti-ALDOA (Fig. 3D). It was worth noting that ALDOA could bind to ACVR2B-as1 as shown by the MS assay (Fig. 3E and F), and other binding proteins could be found in Table S1. Subsequently, utilizing ICC and IHC assays, we observed predominant cytoplasmic localization of ALDOA in human SSCs (Figure S6A-S6B). Furthermore, our RNA-FISH combined with immunofluorescence assays illustrated the cytoplasm co-localization of ACVR2B-as1 and ALDOA in human SSCs (Fig. 3G and H). Taken together, these results strongly suggest that ALDOA is under the regulatory influence of ACVR2B-as1 in human SSCs, highlighting an interaction between ACVR2B-as1 and ALDOA within these cellular contexts.

LncRNA ACVR2B-as1 silencing decreases glycolysis pathway activity in human SSCs

As mentioned earlier, ACVR2B-as1 may regulate glycolytic pathway and interact with ALDOA, a crucial glycolytic enzyme. We conducted a series of experiments in human SSC line to uncover its important role in the glycolytic pathway. Consistently, ECAR assay revealed that ACVR2B-as1 silencing decreased glycolysis in human SSC line (Fig. 4A). ACVR2B-as1 silencing also led to reduction in glucose uptake, lactate generation, and ATP secretion (Fig. 4B and D). Based upon our ChIRP-MS data and relevant literature, [27] we examined several glycolysis-related proteins to see if their expression was altered in human SSC line following the knockdown of ACVR2B-as1. Western blots indicated a dramatic decline in HK2, ENO1, ALDOA, and PGK1 expression levels in human SSC line by ACVR2B-as1 siRNAs (Fig. 4E and I). Considered together, these findings clearly demonstrated that ACVR2B-as1 silencing results in decreases in glycolysis activity in human SSC line.

ALDOA silencing inhibits the proliferation, DNA synthesis and glycolysis and enhances apoptosis of the human SSCs

Recent scRNA-seq of the human testis has shown significantly differential expression of glycolysis-related genes, including *ALDOA*, in SSCs among different age groups [18]. However, the precise function of ALDOA in directing the destiny of human SSCs remains elusive. Three ALDOA siRNAs were designed by us and employed to investigate the role of ALDOA in modulating the fate determinations of human SSC line. Our CCK-8 assay indicated a decline in the number of human SSCs after transfection with all three siRNAs for 4 to 5 days (Fig. 5A). Additionally, there was a consistent reduction in PCNA expression level in the human SSC line after ALDOA siRNAs transfection (Fig. 5B and C). Our EDU incorporation assay revealed that ALDOA knockdown led to a decline in the count of EDU-positive cells in the human SSC line (Fig. 5D and E). Furthermore, ALDOA silencing led to decreases in the levels of glycolysis, glucose uptake, lactate generation, and ATP secretion (Fig. 5F and I). Western blot assays further indicated a dramatic decline in HK2, ENO1, ALDOA, and PGK1

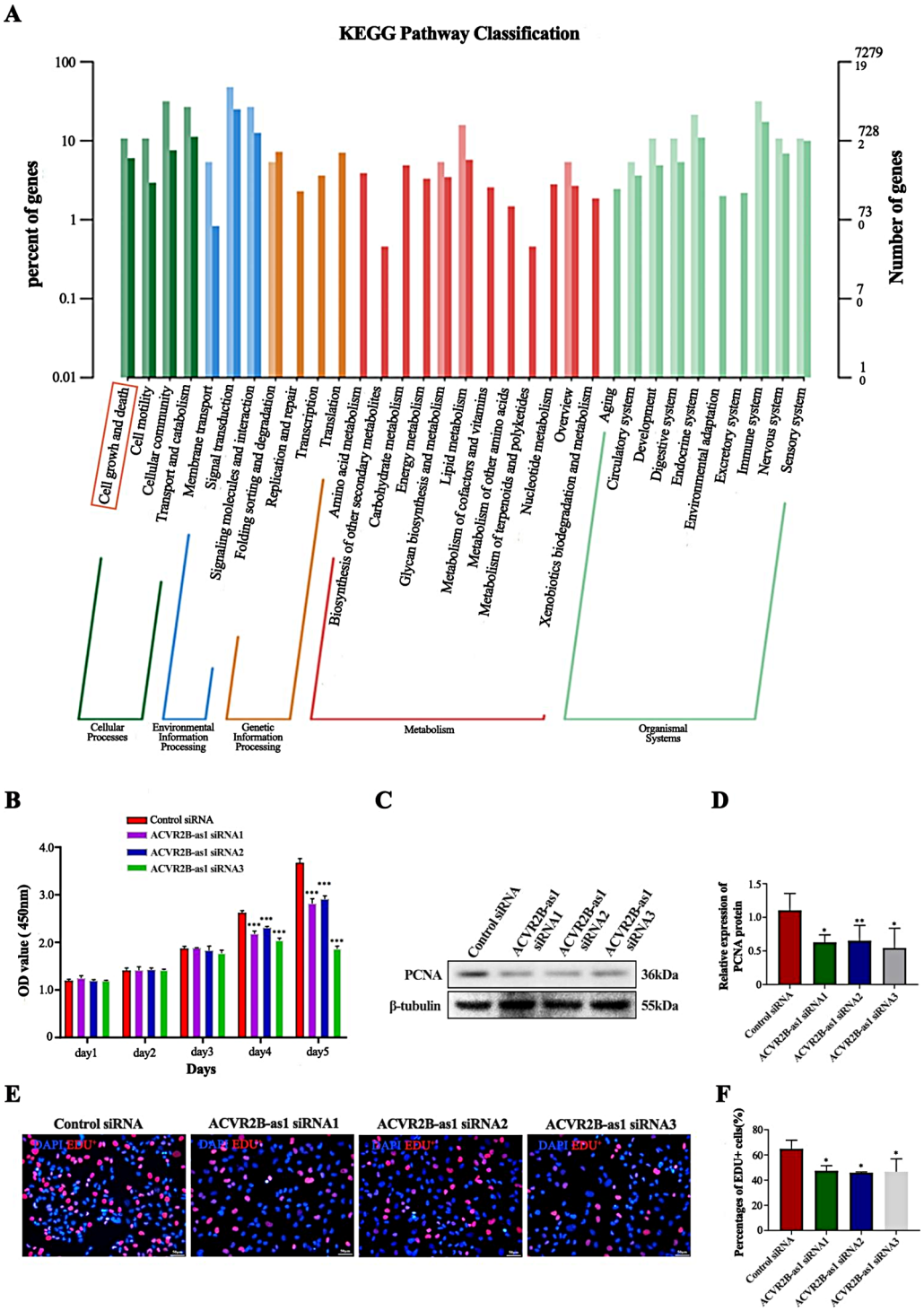

Fig. 2 LncRNA ACVR2B-as1 silencing suppressed the proliferation and DNA synthesis of human SSC line and increased its apoptosis. **A**, KEGG analysis of 231 differentially expressed lncRNAs in human SSC line between between 10% FBS and 0.5% FBS. **B**, CCK-8 assay demonstrated the proliferation ability of human SSC line transfected with the control siRNA and ACVR2B-as1 siRNAs. **C-D**, The expression of PCNA protein in human SSC line transfected with the control siRNA and ACVR2B-as1 siRNAs. **E-F**, The percentages of EDU-positive cells in human SSC line transfected with the control siRNA and ACVR2B-as1 siRNAs. Notes: * indicated $P<0.05$; ** denoted $P<0.01$; *** denoted $P<0.001$

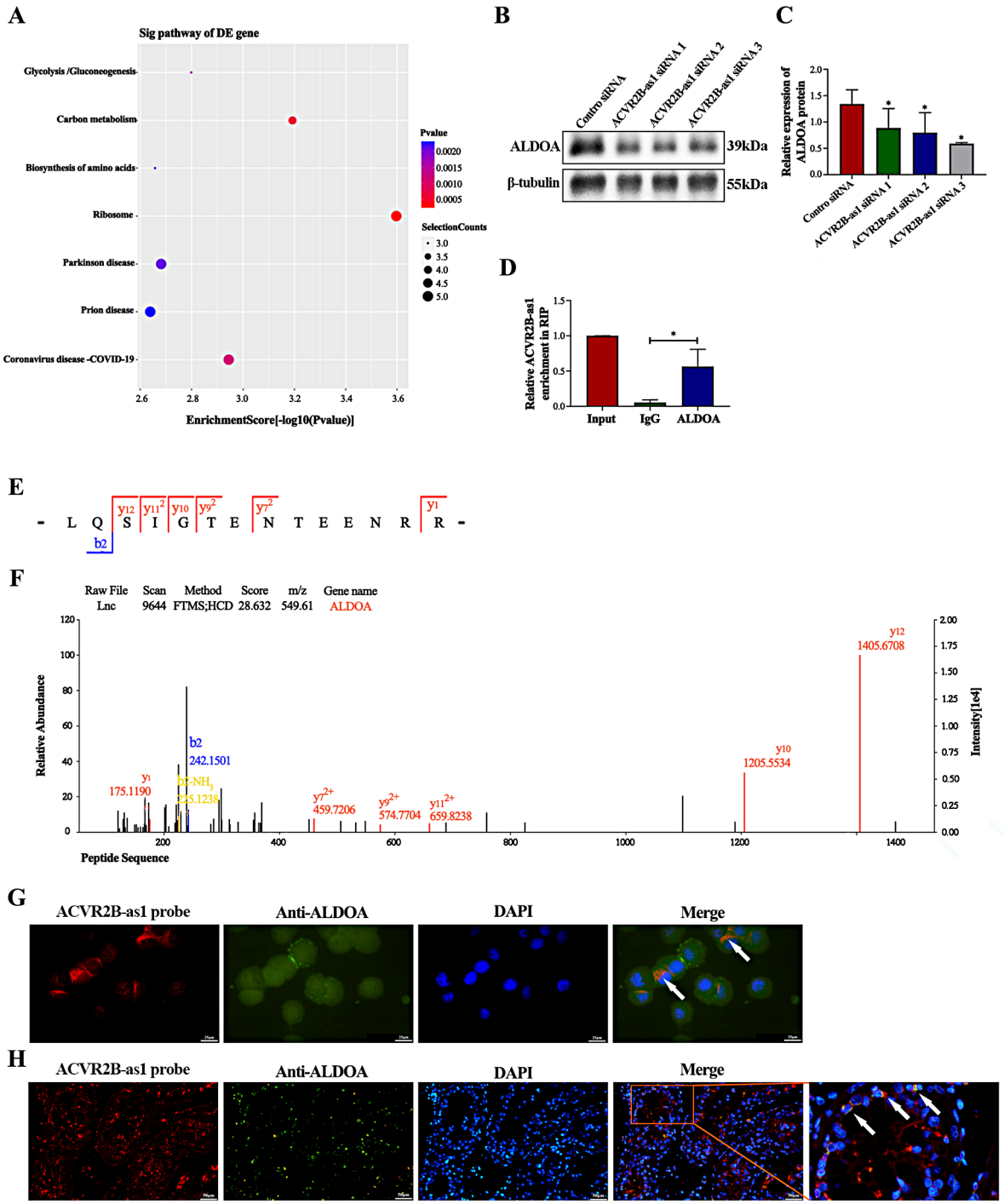

Fig. 3 LncRNA ACVR2B-as1 modulated ALDOA expression through binding in human SSCs. **A**, KEGG analysis of 32 DEGs in human SSC line between the control siRNA and ACVR2B-as1 siRNA. **B-C**, Western blots showed that ALDOA level was decreased by ACVR2B-as1 siRNAs in human SSC line. **D**, The interaction between ACVR2B-as1 and ALDOA protein in human SSC line by RIP. **E**, The ions determined the position of ALDOA in the ChIRP-MS assay of ACVR2B-as1. **F**, Mass spectrum (MS) of ALDOA protein after pulldown in the ChIRP-MS assay of ACVR2B-as1. **G-H** Dual RNA FISH and immunostaining assays showed the co-localization of ACVR2B-as1 (red) and ALDOA (green) in human SSC line (**G**) and human testis (**H**) with DAPI nuclear staining (blue). White arrows showed the positive staining. Notes: * indicated $P < 0.05$

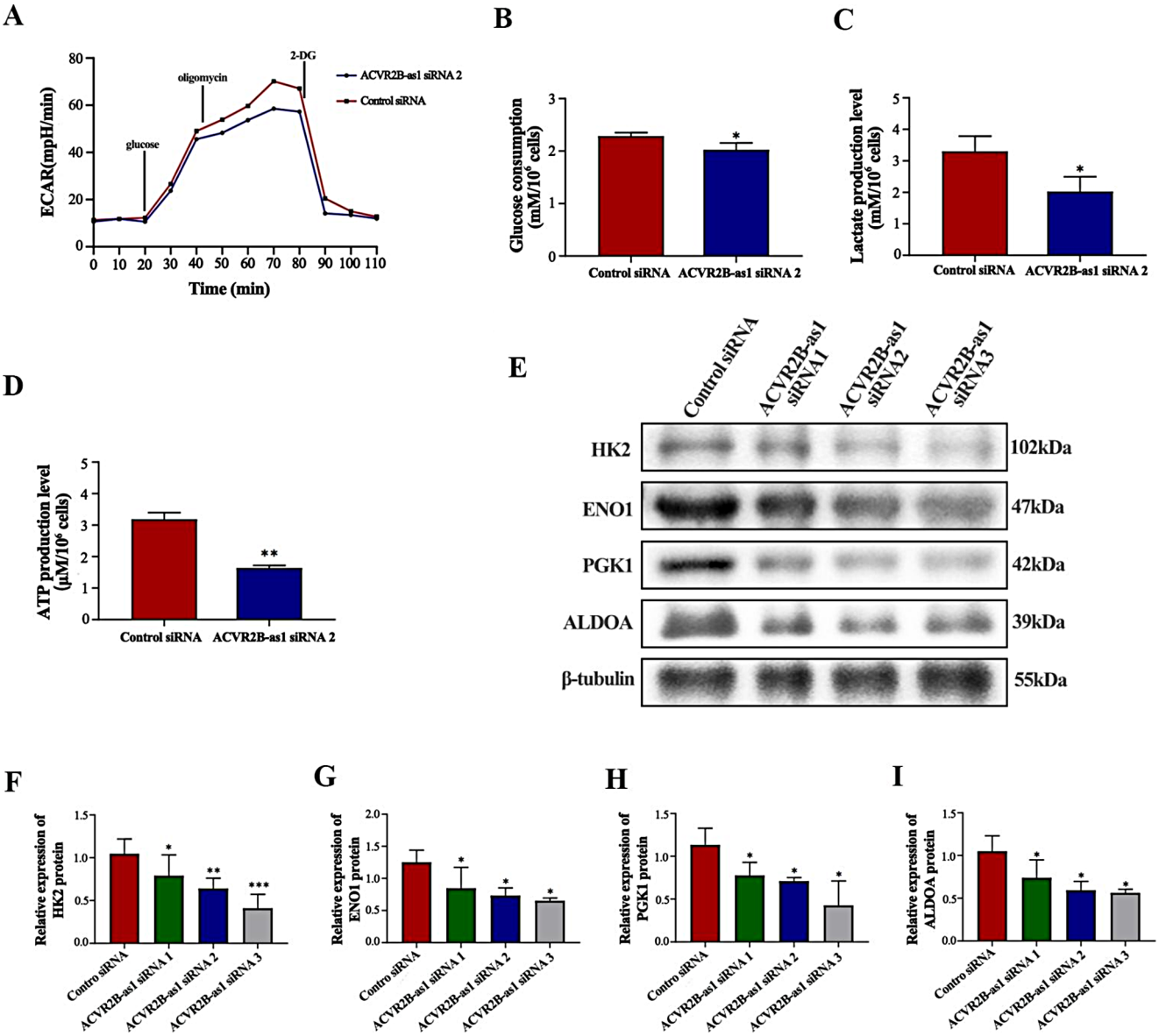

Fig. 4 LncRNA ACVR2B-as1 regulated glycolysis pathway activity in human SSC line. **A**. ECAR assay revealed that ACVR2B-as1 silencing reduced glycolysis in the human SSC line following treatment with glucose (10 mM), oligomycin (1.0 μM), 2-Deoxy-d-glucose (2-DG, 50 mM). **B-C**, The levels of glucose consumption, lactate production, and ATP secretion in the human SSC line treated with ACVR2B-as1 siRNA2 and control siRNA. **E-I**, Western blot analyses of the relative levels of glycolysis-related protein expression in the human SSC line treated with ACVR2B-as1 siRNA1-3 and control siRNA. Notes: * indicated $P < 0.05$; ** denoted $P < 0.01$; *** implicated $P < 0.001$

expression levels (Fig. 5J and N). Moreover, we explored the impact of ALDOA knockdown on apoptosis of human SSC line. As shown in Figure S7, the apoptosis of human SSC line was remarkably elevated by ALDOA siRNAs for 72 h. Taken together, our findings imply that ALDOA silencing results in the reduction in proliferation, DNA synthesis, and glycolysis in human SSCs as well as an increase in apoptosis of these cells.

Glycolysis inhibitor 2-DG (2-Deoxy-d-glucose) reversed the impact of ALDOA overexpression on the proliferation and DNA synthesis of human SSCs

To further explore the regulatory mechanism of ALDOA in human SSCs, we devised an ALDOA overexpression plasmid. Human SSC line infected with ALDOA overexpressing plasmid was subsequently treated with the glycolysis inhibitor 2-DG. As shown in Fig. 6A and C, human SSC line co-transfected with ALCOA-over-expression plasmid and treated with 2-DG exhibited lower expression levels

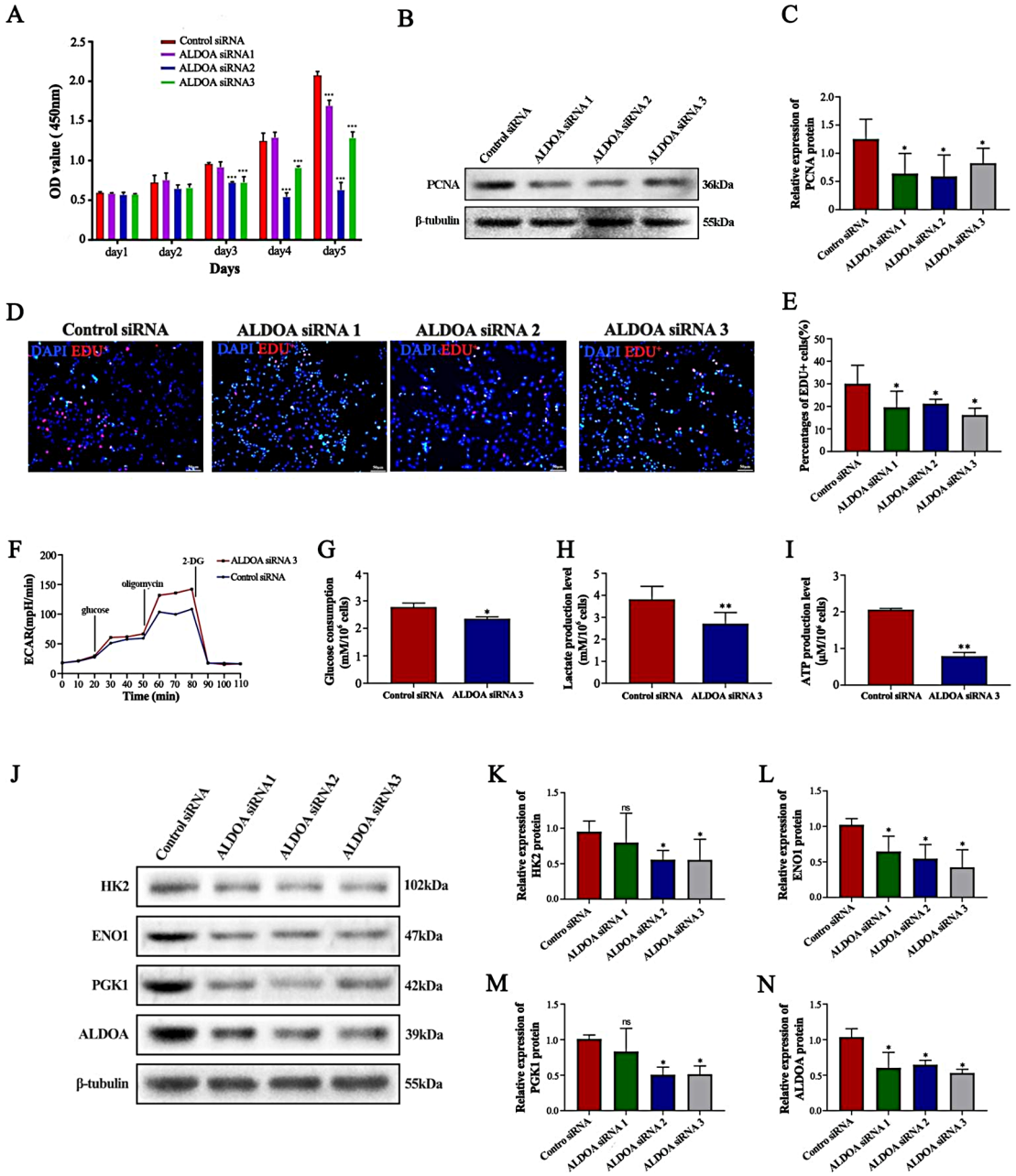

Fig. 5 ALDOA silencing inhibited the proliferation, DNA synthesis, and glycolysis in human SSC line. **A**, CCK-8 assay demonstrated the proliferation ability of human SSC line transfected with the control siRNA and ALDOA siRNA1-3. **B-C**, The expression of PCNA protein in human SSC line transfected with the control siRNA and ALDOA siRNAs. **D-E**, The percentages of EDU-positive cells in human SSC line transfected with the control siRNA and ALDOA siRNAs. **F**, ECAR assay revealed that ALDOA silencing reduced glycolysis in the human SSC line. **G-I**, The levels of glucose consumption, lactate production, and ATP secretion in the human SSC line treated with the control siRNA and ALDOA siRNA1-3. **J-N**, Western blot analyses of the relative levels of glycolysis-related protein expression in the human SSC line transfected with the control siRNA and ALDOA siRNA1-3. Notes: * indicated $P < 0.05$; ** denoted $P < 0.01$

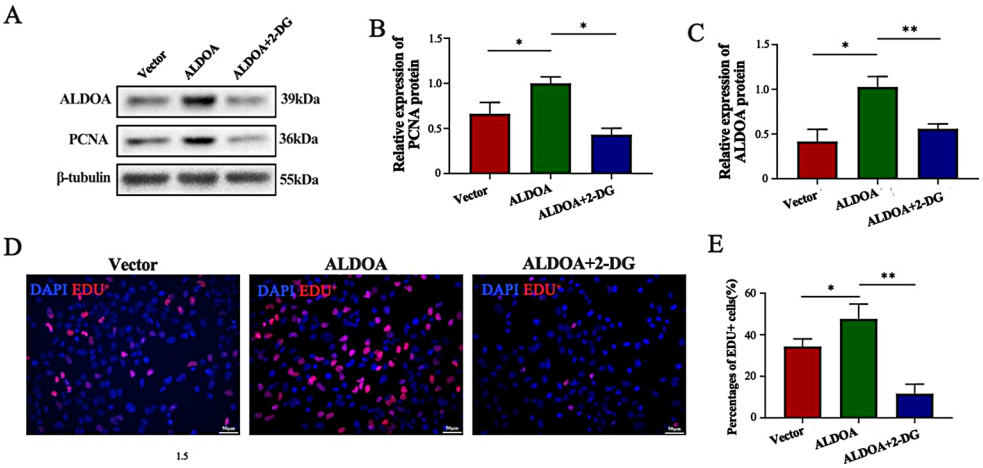

Fig. 6 Glycolysis inhibitor 2-DG reversed the impact of ALDOA over-expression on proliferation and DNA synthesis of human SSC line. **A-C**, Western blot analysis of the relative levels of ALDOA and PCNA expression in human SSC line treated with or without 2-DG and/or ALDOA overexpression plasmid. **D-E**, EDU incorporation assay was employed to evaluate the DNA synthesis capacity in human SSC line treated with or without 2-DG and/or ALDOA overexpression plasmid. Notes: * indicated $P < 0.05$; ** denoted $P < 0.01$

of PCNA and ALDOA, which were elevated by ALDOA overexpression. Furthermore, consistent with these results, ALDOA overexpression combined with 2-DG treatment decreased the percentages of EDU-positive cells in human SSC line in comparison to ALDOA overexpression alone (Fig. 6D and E**)**. Collectively, these data implicate that the role of ALDOA in controlling the proliferation and DNA synthesis of human SSCs might be reliant on the glycolysis pathway.

ALDOA is involved in the impact of LncRNA ACVR2B-as1 on proliferation, DNA synthesis, and glycolysis of human SSCs

We further assessed the role of ALDOA in the enhancement of proliferation, DNA synthesis, and glycolysis by ACVR2B-as1 in human SSCs. Thus, we induced overexpression of ALDOA in ACVR2B-as1 silencing human SSC line. Interestingly, ALDOA overexpression could counteract the inhibitory effect of ACVR2B-as1 on proliferation (Fig. 7A) and DNA synthesis (Fig. 7B, C, D and E). Additionally, ECAR assays exhibited that ALDOA overexpression abolished the decrease in glycolysis caused by ACVR2B-as1 silencing (Fig. 7F). A similar trend was evident in the measurements of glucose uptake, lactate generation, and ATP secretion (Fig. 7G, H, and I). Western blot displayed that silencing of ACVR2B-as1 resulted in a reduction in the expression levels of HK2, ENO1, ALDOA, and PGK1, which were alleviated or nullified by ALDOA over-expression (Fig. 7J, K, L, M and N). Moreover, apoptotic cells of human SSC line were decreased by ALDOA overexpression plasmid andACVR2B-as1 siRNA2 (Fig. S8). Together, these results demonstrated that ACVR2B-as1 mediates the proliferation, DNA synthesis, and glycolysis through its regulation of ALDOA in human SSCs.

Materials and methods

Cell culture and siRNA transfection

The human SSC line was established by us, and it possesses the biochemical phenotypes of primary human SSCs. This cell line was thus utilized in this study and named human SSCs. For cell culture, human SSCs were incubated with DMEM/F12 medium with 10% FBS (Gibco, 10099-141) and PS (Gibco, 15140-122) in 5% CO_2 incubator [28]. We utilized 10% FBS to stimulate the proliferation of human SSC line, and we sought to identify the novel lncRNAs upregulated by 10% FBS with an aim to promote human SSC proliferation. For siRNA transfection, cells were seeded onto dishes and transfected with siRNAs synthesized by Genepharma (Suzhou, China) using Lipofectamine 3000 reagent (Cat No. L3000015, Invitrogen, CA, USA), and siRNA sequences were shown in Table S2. Gene expression was assessed at 48 h post-transfection, while protein analyses were conducted at 72 h post-transfection.

Acquirement of human testis tissues

Human testicular tissues, obtained from patients undergoing orchiectomy at Hunan Cancer Hospital, were washed thrice with PBS containing 4% PS. They were subsequently fixed by Bouin's or preserved with storing in liquid nitrogen. This study was approved from the Third Xiangya Hospital of Central South University's Ethical Review Committee. Patients consented to the use of their tissues only for scientific investigation and signed consent forms with informed content.

Immunocytochemistry (ICC)

Cells were collected by centrifugation and fixed with 4% PFA. After three PBS washes, cells were treated with 0.25%

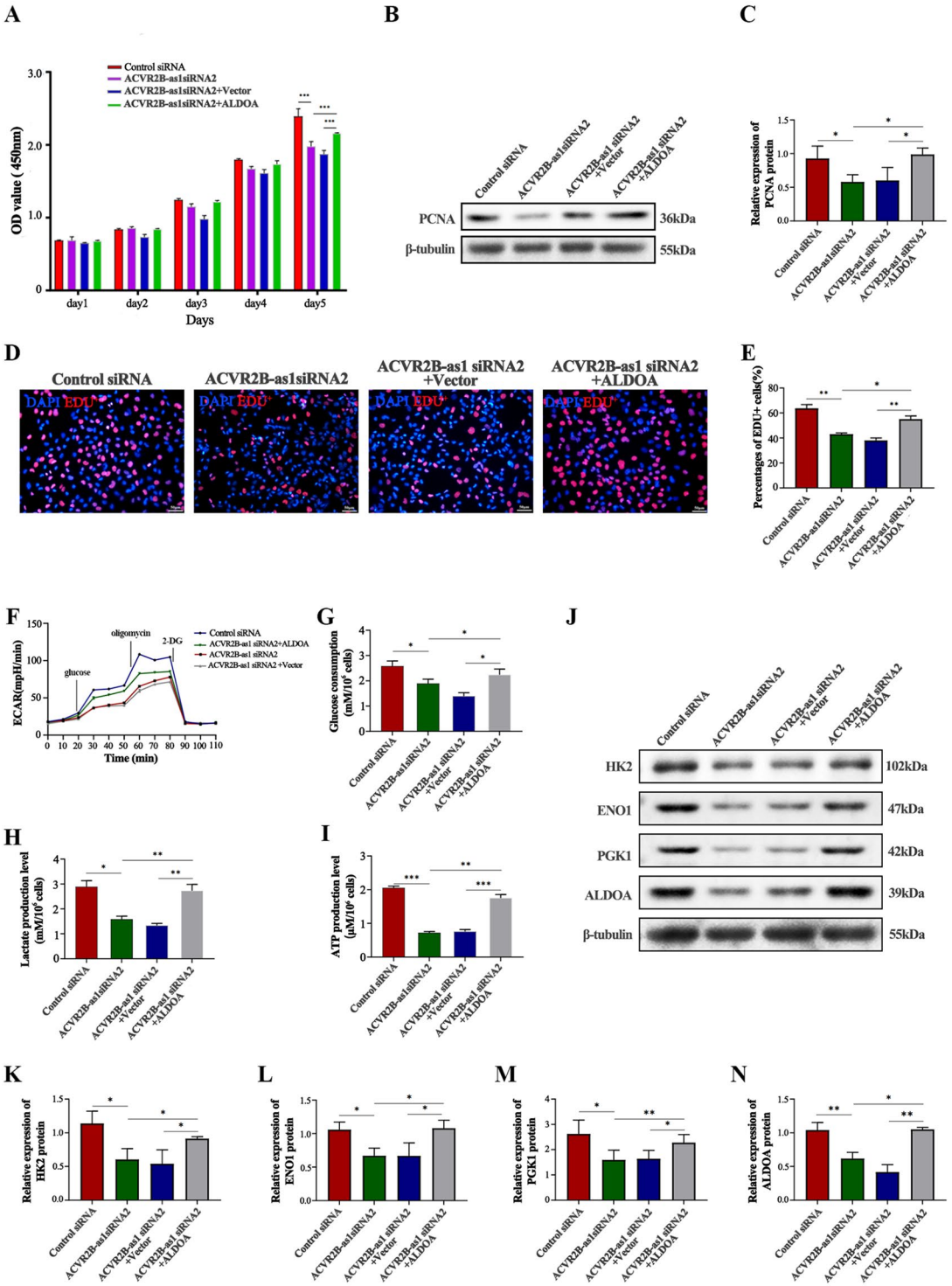

Fig. 7 ALDOA is involved in the regulation of LncRNA ACVR2B-as1 in proliferation, DNA synthesis, and glycolysis of human SSC line. **A**, CCK8 assay were employed to evaluate the proliferation capacity of human SSC line treated with ACVR2B-as1 siRNA2, control siRNA, and ALDOA overexpression plasmid. **B-C**, Western blot analysis of the relative levels of PCNA expression in human SSC line treated with ACVR2B-as1 siRNA2, control siRNA, and ALDOA overexpression plasmid. **D-E**, EDU incorporation assay was employed to evaluate the DNA synthesis capacity in human SSC line treated with or without 2-DG and/or ALDOA overexpression plasmid. **F**, ECAR assay revealed the glycolysis activity in human SSC line treated with ACVR2B-as1 siRNA2, control siRNA, and ALDOA overexpression plasmid. **G-I**, The levels of glucose consumption, lactate production, and ATP secretion in human SSC line treated with ACVR2B-as1 siRNA2, control siRNA, and ALDOA overexpression plasmid. **J-N**, Western blot analyses of the relative levels of glycolysis-related protein expression in human SSC line treated with ACVR2B-as1 siRNA2, control siRNA, and ALDOA overexpression plasmid. Notes: * indicated $P < 0.05$; ** denoted $P < 0.01$; *** implicated $P < 0.001$

Triton X-100 and blocked by BSA at room temperature (RT). Cells were incubated with primary antibodies (Table S3), and after washing with PBS, they were incubated with secondary antibodies (Table S4). Cell nuclei were labelled with DAPI, and immunostaining was visualized under a microscope (Leica, DM3000, Germany).

Immunohistochemistry (IHC)

Human testicular tissue sections were dewaxed and dehydrated by gradient alcohol, and endogenous peroxidase was inactivated with 3% H_2O_2. After washing with PBS, sections were blocked with BSA and incubated with primary antibodies (Table S3). For immunofluorescence staining, these sections were incubated with secondary antibodies (Table S4), and cell nuclei were labelled with DAPI. Images were captured by microscope (Leica, DM3000, Germany).

Western blot

Cells were lysed using RIPA buffer, and cell lysis was centrifugated at 12,000 rpm. The concentrations of proteins were determined by the BCA kit. Proteins were resolved on SDS-PAGE gels and transferred onto PVDF membranes (Millipore, Immobilon-P, IPVH00010). Membranes were treated with QuickBlock buffer (Beyotime, P0252) for blocking and then incubated overnight with primary antibodies (Table S3) and followed by secondary antibodies (Table S4). Blots were visualized by ECL Kit (GENVIEW and GE2301).

RT & quantitative PCR (qPCR) and RT-PCR

Total RNA was extracted from cells using TRIzol reagent (Vazyme, R401-01), and the quality and concentrations of total RNA were measured by Nanodrop (Thermo Fisher Scientific, USA). The cDNAs were synthesized utilizing EvoMMLVRT Premix (Accurate Biology, AG11706) and followed by qPCR analysis using a qPCR Kit (AG11701, Accurate Biotechnology, Hunan, China) on the CFX Connect™ Fluorescent Quantitative PCR Detection System (Bio-Rad, USA). The relative expression levels of the target genes in the treatment and control groups were calculated using the formula $2^{-\Delta\Delta Ct}$ [$\Delta\Delta Ct = \Delta Ct$ (treatment group) - ΔCt (control group)].

RT-PCR was carried out utilizing 2×Taq Master Mix (Dye Plus) (Vazyme, P222-AA) and followed by electrophoresis. Gel images were obtained by a Gel Documentation and Image Analysis System (ChampGel 5000).

CCK-8 assay

The CCK-8 (Apexbio, K1018) was used to assess cell proliferation of human SSCs. Cells were seeded onto 96-well culture dishes and cultured for 5 days. CCK-8 reagent was added to cells that were cultured for 1 to 5 days. The absorbance at 450 nm was measured using a reader (Biotek, Synergy 2).

EDU incorporation assay

Human SSCs were cultured with DMEM/F12, and EDU reagent A was added to the cells for 12 h. Cells were fixed by 4% PFA and treated with glycine. The Apollo solution was used for staining and followed by DAPI staining. EDU-positive cells were visualized under a microscope (Leica, DMi8, Germany) and the percentages of EDU-positive cells were counted by (EDU^+ cells/$DAPI^+$ cells)×100%.

Flow cytometry

Cell apoptosis was evaluated by flow cytometry utilizing a Kit with PI staining (Biolegend,640932). After washing twice with PBS, they were resuspended in 100 µl of Annexin V Binding Buffer. Subsequently, cells were treated with 5 µl APC Annexin V and 10 µl Propidium Iodide (PI) Solution and followed by incubation at room temperature in the dark for 15 min. The reaction was terminated by adding 400 µl Annexin V Binding Buffer. Flow cytometry (FACSCanto II 488 N, BD Bioscience, USA) was employed for the detection of apoptosis of cells.

TUNEL assay

Cell apoptosis was also assessed using the Kit (Servicebio, G1502). Cells were fixed with 4% PFA for 25 min and permeabilized using 0.5% Triton X-100 for 20 min or 20 µg/ml proteinase K for 4–6 min. Subsequently, cells were incubated with the labeling reaction mixture containing Cy3-dUTP at room temperature in the dark for 1 h. Cell nuclei were labeled with DAPI, and TUNEL-positive cells were visualized under a fluorescence microscopy (Leica, DMi8, Germany).

Plasmid construction and transfection

To overexpress ALDOA, full-length ALDOA was ligated into a GV741 vector (Genechem, Shanghai, China). The corresponding negative control was also obtained from Genechem. After human SSC line was cultured at 50–65% confluence, Lipofectamine 3000 Reagent (Cat No.

L3000015, Invitrogen, CA, USA) was used for transfection of ALDOA overexpression plasmid.

RNA sequencing (RNA-seq)

RNA-seq and analysis were performed by Sangon Biotech Co., Ltd. (Shanghai, China). Total RNA was extracted and RNA sequencing was carried out on an Illumina HiSeqTM 2000 (San Diego, CA, USA). For the analysis of the differentially expressed lncRNAs, the DESeq2 R package with $P < 0.05$ and |Fold change|≥2 was employed. Hierarchical clustering was used to unveil gene expression patterns, while R-based hypergeometric distribution was utilized for pathway enrichment analysis.

RNA fluorescence in situ hybridization (FISH) assay

To visualize the subcellular distribution of LncRNA ACVR2B-as1 in human SSCs, RNA FISH Kit (Genepharma Suzhou, China) was employed. Probes were designed and synthesized by Genepharma and they were hybridized overnight at 42°C with enough humidity. Probes used for detecting LncRNA ACVR2B-as1 were listed in Table S5.

Isolation of cytoplasmic and nuclear RNA

Nuclear and cytoplasmic RNA was extracted from human SSCs utilizing Kit (Life Technology). Actin and small nuclear RNA U6 served as transcription controls, respectively.

ChIRP- Mass spectrometry (MS)

Formaldehyde cross-linking immobilized intracellular RNA-protein and RNA-nuclear acid complexes binding to RNA were isolated. Mass spectrometry (MS) was employed to identify proteins in the separated products, which enables the screening of proteins binding to the target RNAs. Seventeen Biotin-TEG-modified LncRNA ChIRP probe was synthesized and hybridized with the cross-linked LncRNA molecular complex. The associated chromatin complex was isolated, while proteins purified from the complex were fragmented into peptide mixtures by a protease and then analyzed. Mass spectrometric peak maps were generated to identify proteins. Probes used for ChIRP assay were described in Tables S5.

RNA immunoprecipitation (RIP) assay

A commercial RIP kit (BersinBio, Guangzhou, China) and antibodies against IgG (BersinBio, Guangzhou, China) and AGO2 (FNab10000; FineTest, Hubei, China) were utilized. Human SSCs were lysed with NP-40 Lysis Buffer (Solarbio) and centrifuged, and the supernatant was removed. The beads were risen prior to proteinase K digestion. RNA was isolated and analyzed with primers for detecting LncRNA ACVR2B-as1 as listed in Table S6.

Agarose gel electrophoresis

Agarose gels were prepared by dissolving agarose powder (BaygeneBio, Shanghai, China) in TAE buffer solution (Solarbio, Beijing, China) and mixing with GelRed (Mei5Bio, Beijing, China). Electrophoresis was conducted at 100 V for 45 min using a 1% TAE solution. Gene products were visualized and analyzed utilizing Image Lab software (Bio-Rad, Hercules, CA, United States).

Assessment of glycolysis, glucose consumption, lactate production, and ATP secretion levels

After siRNA transfection, human SSCs at 1×10^6 cells/well were incubated for 2 days. Glycolytic activity was measured using Extracellular Acidification Rate Assay Kit (BB-48311, BestBio, Shanghai, China). Briefly, cells were cultured in the conditioned medium under CO_2 free conditions for 1 h and calibrated. Subsequently, glucose, oligomycin, and 2-DG (Sigma) were added to cells, and the ECAR was measured. Glucose, lactate levels, and ATP secretion were determined by the Glucose Colorimetric Assay Kit, Lactate Assay Kit, and ATP Assay Kit (E-BC-F037, E-BC-K044-M, and E-BC-F002, Elabscience, Wuhan, China), respectively.

Statistical analysis

Data were presented as the means ± SD. GraphPad Prism 8.0. were utilized, and The t-test was used to determine statistical differences between two groups. Risk analysis was performed using the chi-square test and logistic regression. Statistical significance was defined as $P < 0.05$.

Discussion

It has been reported that ACVR2B-as1 plays an important role in several cancer prognosis [29, 30]. For example, ACVR2B-as1 has been regarded as a potential biomarker for liver cancer prognosis [31]. Nevertheless, the exact functions and mechanisms of ACVR2B-as1 in controlling fate decisions of human SSCs await to be explored. We observed an elevated expression level of ACVR2B-as1 in human SSC line by 10% FBS, and significantly, we have demonstrated that ACVR2B-as1 interacts with ALDOA to

regulate the proliferation, DNA synthesis, glycolysis, and apoptosis of human SSCs.

Glycolysis emerges as a pivotal regulator of SSC self-renewal [15]. Prior investigations have underscored that adult human and mouse SSCs exhibit enrichment for glycolysis-related genes, which concurrently upregulates the OXPHOS-associated genes during spermatogenesis [32–35]. Furthermore, it has been demonstrated that optimizing the conditions to promote glycolysis as the main bioenergetic process significantly enhances the regenerative health of both young and mature SSCs. Notably, this optimization leads to a remarkable 100% efficiency of establishing primary SSC culture. The results indicate that SSCs are inclined towards conditions that promote glycolytic activity, highlighting the importance of aligning culture environments with their bioenergetics requirement to maintain SSC functional integrity [16]. Notably, both adult human and mouse SSCs have been reported to rely on glycolytic metabolism for the preservation of stem cell-specific signaling [17]. In our study, we observed that the glycolysis inhibitor 2-DG reversed the impact of ALDOA overexpression on the proliferation and DNA synthesis of human SSC line. The outcomes were consistent with prior discoveries highlighting the critical role of glycolysis in SSC function. Additionally, our findings propose that the increased glycolytic activity stimulated by ALDOA can be responsible for the increases in the proliferation and DNA synthesis of human SSCs.

ALDOA plays a critical role in glycolysis [25]. We demonstrated that ALDOA knockdown led to reduction in the proliferation, DNA synthesis, and glycolysis of human SSC line, accompanied by an elevation in apoptosis. Interestingly, we noted a parallel expression pattern between ACVR2B-as1 and ALDOA in human SSC line. Furthermore, manipulating ACVR2B-as1 expression modulated ALDOA expression in human SSC line. Through ChIRP-MS and RIP assays, we elucidated an interaction between ALDOA and ACVR2B-as1, highlighting that ACVR2B-as1 could pull down ALDOA, and conversely, ALDOA could bind to ACVR2B-as1 in human SSCs. Furthermore, we revealed that ALDOA overexpression could counteract the impact of ACVR2B-as1 knockdown on the proliferation, DNA synthesis, glycolysis, and apoptosis of human SSCs. Collectively, our findings implicate that ALDOA acts as a downstream target of ACVR2B-as1 to modulate fate decisions of human SSCs through the glycolysis pathway. To our understanding, this marks the first evidence showing that ACVR2B-as1 triggers ALDOA expression to foster the self-renewal of human SSCs. In summary, our findings implicate that LncRNA ACVR2B-as1 mediates the self-renewal and apoptosis of human SSCs via an interaction with ALDOA to control glycolysis activity. This study thus provides a new regulatory mechanism by epigenetic factors to control the fate determinations of human SSCs.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00018-024-05414-w.

Acknowledgements This work was supported by the grants from the National Nature Science Foundation of China (32170862 and 31872845), Major Scientific and Technological Projects for Collaborative Prevention and Control of Birth Defect in Hunan Province (2019SK1012), Developmental Biology and Breeding (2022XKQ0205), the Graduate Self-Exploration and Innovation Project of Central South University of China (2023ZZTS0549), Research Team for Reproduction Health and Translational Medicine of Hunan Normal University (2023JC101), and the Shanghai Key Laboratory of Reproductive Medicine. Competing interests: The authors declare that they have no competing interests.

Funding This work was supported by the grants from the National Nature Science Foundation of China (32170862 and 31872845), Major Scientific and Technological Projects for Collaborative Prevention and Control of Birth Defect in Hunan Province (2019SK1012), Developmental Biology and Breeding (2022XKQ0205), the Graduate Self-Exploration and Innovation Project of Central South University of China (2023ZZTS0549), Research Team for Reproduction Health and Translational Medicine of Hunan Normal University (2023JC101), and the Shanghai Key Laboratory of Reproductive Medicine.

Data availability The data are available upon the request from the corresponding authors.

Declarations

Competing interests The authors declare no competing interest.

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