Oncology Treatment Discovery, 2026, Volume 4, Issue 2
http://ojs.bbwpublisher.com/index.php/OTD
Online ISSN: 2981-8079
Print ISSN: 3083-4996

Transcriptional Repression of SP1 by FOXA3 Suppresses Pancreatic Cancer Cell Proliferation and Migration

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Abstract: *Objectives*: Transcriptional Repression of SP1 by FOXA3 Suppresses Pancreatic Cancer Cell Proliferation and Migration. *Methods*: Using overexpression and RNAi (RNAi) techniques, the expression levels of SP1 and FOXA3 in pancreatic cancer cells were adjusted, respectively. Cell proliferation and migration abilities were assessed by CCK - 8 proliferation assay and wound healing assay. The regulatory relationship between SP1 and FOXA3 expression was confirmed by Western blot analysis. *Results*: Functional assays showed that knockdown of SP1 significantly suppressed the proliferation and migration abilities of pancreatic cancer cells, indicating an oncogenic role of SP1 in pancreatic cancer. Mechanistic studies further demonstrated that overexpression of FOXA3 markedly downregulated the protein expression level of SP1. *Conclusion*: By transcriptionally suppressing SP1 expression, FOXA3 prevents the malignant progression of pancreatic cancer and attenuates SP1- mediated promotion of tumor cell proliferation and migration. Targeting the FOXA3/SP1 regulatory axis may offer a novel therapeutic approach for pancreatic cancer treatment.

Keywords: SP1; FOXA3; Proliferation; Migration

Online publication: June 30, 2026

1. Introduction

Pancreatic cancer is one of the most deadly cancers of the digestive system, with a steadily increasing incidence worldwide [1]. Due to its insidious early symptoms and lack of specific biomarkers, the majority of patients are diagnosed at locally advanced stages or with distant metastases, missing the opportunity for curative surgical resection [2]. Although some progress has been made in recent years in chemotherapy (e.g., agents targeting KRAS G12C, NTRK, MSI-H), Pancreatic cancer typically presents primary or acquired resistance to existing treatment modalities [3–5]. The 5-year survival rate remains stagnant at approximately 10%, indicating a very poor prognosis [6]. The high invasiveness of pancreatic cancer, Various desmoplastic

stromal reactions. Additionally, the uniquely immunosuppressive tumor microenvironment represents critical pathological features contributing to its aggressive progression and therapeutic resistance [7]. Therefore, in-depth explanation of important molecular events during pancreatic carcinogenesis and progression, and clarification of the transcriptional regulatory networks underlying proliferation, migration, and drug resistance, holds important theoretical, clinical and hypothetical value for finding new diagnostic biomarkers and efficient therapeutic targets.

SP1 (Specificity Protein 1), as a crucial transcription factor, plays a multifaceted regulatory role in the occurrence, progression, metastasis, metabolic reprogramming, and therapeutic resistance of various cancers. In terms of boosting tumor proliferation and progression, in hepatocellular carcinoma (HCC), SP1 mediates the regulation of LOXL2 by ZRANB1. Overexpression of SP1 reverses the suppression of growth and metastasis caused by ZRANB1 knockdown. Mechanistically, ZRANB1 directly binds to SP1 and stabilizes its protein level through deubiquitination [8]. In cisplatin-resistant colon adenocarcinoma cells, SP1 and GOLPH3 are co-overexpressed; knockdown of SP1 upregulates NDRG1, inhibits cell proliferation, promotes apoptosis, and improves chemosensitivity [9]. In regulating epithelial - mesenchymal transition (EMT) and metastasis, pan - cancer analysis confirms SP1 as a major driver of metastasis, promoting cancer cell survival, invasive growth, and metastatic colonization in multiple cancer types [10]. In prostate cancer, SP1 has been identified as a key regulator of EMT: its overexpression promotes EMT in vitro and boosts the invasive capacity of PC3 cells [11], but in vivo studies demonstrate that it inhibits lung metastasis, suggesting a microenvironment - dependent role. In lung cancer, SP1 functions as a downstream target of the Hippo - TEAD1 pathway and directly binds to the EHD1 promoter to promote its expression [12]. Clinical samples demonstrate significantly higher EHD1 expression in tissues from patients with metastatic lung adenocarcinoma. However, the mechanistic role of SP1 in pancreatic cancer remains unclear.

SP1 is aberrantly expressed in pancreatic cancer, according to our research. Additionally, we discovered a new mechanism controlling SP1, which was validated by preliminary validation of the upstream regulators of SP1 and the effects of knockdown of SP1 on tumor function in vitro.

2. Materials and methods

2.1. Cell lines

The Chinese Academy of Sciences' Shanghai Cell Bank provided the human pancreatic cancer cell lines HPNE, MIA PaCa - 2, and PANC - 1. These cells were kept in DMEM medium (Shanghai Yuanpei Biotechnology Co., Ltd.) supplemented with 10% fetal bovine serum (Wuhan Servicebio Technology Co., Ltd.), LTD (Ltd), and 5%100 penicillin (streptomycin), amphotericin B (Wuhan Servicebio Technology Co.), and 5%100 penicillin. In an incubator with 5% CO_2, MIA PaCa - 2 and PANC - 1 cells were cultivated at 37 ℃ and kept as monolayer cultures. Cells were subjected to experiments at roughly 70–80% of the influence.

2.2. Cellular RNA extraction

2.2.1. Lysis of adherent cells

(1) Aspirate the culture medium from the dish and wash the cells once with 1× PBS buffer.

(2) Remove the PBS buffer, then add 500 μL of Buffer QLS lysis solution to no more than 1.0×10^7 cultured cells. Gently shake the culture dish to ensure the lysis solution is evenly distributed across the cell surface.

(3) Repeatedly pipette to separate the cells, then transfer the complete cell lysate to a centrifuge tube.
(4) Vortex vigorously or pipette repeatedly for 30 seconds to 2 minutes, until the lysate becomes clear and non-viscous. (Note: Generally, for complete lysis, vigorous vortexing or repeated pipetting for 30 seconds to 2 minutes is sufficient. If the sample stays viscous, the lysis time can be suitably increased.)
(5) Let the lysate stand at room temperature for 2 minutes.

2.2.2. Purification steps

(1) Add a 100% ethanol to the lysate mentioned above and mix it by pipetting. If the precipitate or viscous material is visible, the pipette will be repeated several times to break up the precipitate. (Note: The Quick RNA Mini Columns may be clogged by the inability to break up the precipitate, which could impact yield and purity.)
(2) Immediately transfer the entire mixture to a Quick RNA Mini Column. Centrifuge at 12,000 rpm for 2 minutes at room temperature, then discard the flow-through.
(3) Add 700 μL of Buffer QWB to the Quick RNA Mini Column. Centrifuge at 12,000 rpm for 1 minute at room temperature, and discard the flow-through. (Note: Ensure that 100% ethanol has been added to Buffer QWB in the specified volume.)
(4) Place the Quick RNA Mini Column into a new 2.0 ml Collection Tube and centrifuge at 12,000 rpm for 2 minutes at room temperature.
(5) Transfer the Quick RNA Mini Column to a new RNase-Free Tube. Add 30 μL to 200 μL of RNase-Free Water to the center of the column membrane. Allow it to stand at room temperature for 3 minutes, then centrifuge at 12,000 rpm for 2 minutes at room temperature to elute the RNA. Store the eluted RNA at -80℃.

2.3. Reverse Transcription PCR

The extracted RNA was removed from the -80℃ freezer. After thawing, 1 μL of RNA was placed in a spectrophotometer to measure its concentration. Based on the concentration, the necessary volume was determined. For each sample, 3000 ng of RNA was taken, mixed with 2 μg of random primers, and DEPC-treated water was added to a final volume of 35 μL. A standard PCR device was used to heat the mixture for five minutes at 70 degrees Celsius. During this period, a 15 μL reaction mixture was made, containing 10 μL M - MLV buffer, 2 μL 4 dNTPs, 2μL M - MLV reverse transcriptase, and 1 μL RNase inhibitor per sample, for a total volume of 15 μL. After the heating step, the 15 μL mixture was added to each sample, followed by incubation at 37 C for 60 minutes and then at 70℃ for 10 minutes. Following reverse transcription, the resulting cDNA was stored in a -20℃ freezer.

2.4. Real-time quantitative PCR (qPCR)

(1) Preparation of the qPCR reaction mixture: For each sample, take 1 μL of cDNA, then add 5 μL of SYBR Green, 3 μL of double-distilled water, and 1 μL of primers. After mixing, centrifuge the mixture in a balanced centrifuge at 1000 rpm for 1 minute.
(2) qPCR program: 10 minutes of dehydration at 95℃, followed by 40 amplification cycles: 15 seconds at 95℃, 30 seconds at 60℃, and 35 seconds at 72℃. A final extension step is performed at 72℃ for 10 minutes.

(3) Three replicate wells were set up for each group, and the CT value difference within each group did not exceed 1. If the difference exceeded 1, the experiment was repeated.

(4) Calculation:

ΔCT = CT value of target gene – CT value of internal control

$\Delta\Delta CT$ = ΔCT value of experimental group – ΔCT value of control group

Finally, $2^{-}\Delta\Delta CT$ is obtained.

(5) **Interpretation:**

If $2^{-}\Delta\Delta CT$ is greater than 1, it indicates that the expression level of the target gene in the experimental group is increased compared with the control group.

If $2^{-}\Delta\Delta CT$ is less than 1, it indicates that the expression level of the target gene in the experimental group is decreased compared with the control group.

2.5. Plasmid transfection

For plasmid transfection, small interfering RNA (siRNA) targeting Sp1 and overexpression RNA (oe-RNA) targeting FOXA3 were obtained from Shanghai Genechem Co., Ltd. (Shanghai, China). Cells were seeded in appropriate culture plates and allowed to reach the optimal confluence for transfection according to the manufacturer's recommendations. Transfection was performed using the siRNA transfection reagent jetPRIME (Polyplus Transfection, France) following the supplier's protocol. Briefly, the siRNA or oeRNA was mixed with the jetPRIME reagent and added dropwise to the cells. The transfected cells were then incubated under standard culture conditions for 24 hours before subsequent experiments.

2.6. Western blot

2.6.1. Sample preparation

Total protein was extracted from cells using IP lysis buffer (QIAGEN) supplemented with a protease inhibitor (Shanghai Beyotime Biotechnology Co., Ltd.) at a ratio of 100:1. Protein concentrations were measured using the BCA protein assay kit (Thermo Fisher Scientific). The protein samples were then mixed with loading buffer (Thermo Fisher Scientific) and boiled to achieve denaturation.

2.6.2. SDS-PAGE electrophoresis

Equal volumes of protein were loaded into the gel's wells, and electrophoresis was carried out at a constant voltage of 80 V until the molecules were fully separated.

2.6.3. Transfer

Using a " sandwich" structure (sponge-filter paper - gel - membrane - filter paper - sponge) and an electric field, the proteins on the gel were transferred onto a nitrocellulose (NC) membrane.

2.6.4. Blocking

For two hours, the membrane was blocked with 20% non-fat milk.

2.6.5. Antibody incubation

After blocking and washing, the membrane was incubated with a specific primary antibody against the target protein at 4℃ for 12 hours to allow specific binding. All primary antibodies used in this experiment were

purchased from Wuhan Proteintech Biotechnology Co., Ltd. Following primary antibody incubation, the membrane was washed thoroughly and then incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature.

2.6.6. Signal detection and imaging

A chemiluminescent substrate was added to react with the HRP label on the secondary antibody, producing luminescence. Images of the resulting black bands were captured using a gel imaging system.

2.7. CCK-8 Cell Proliferation Assay

Cells were digested and counted. A total of 8×10^3 cells per well were seeded into 96-well plates, with 6 rows and 5 columns for each cell type. After 24 hours, the culture medium in each well was adjusted to 200 μL, and the absorbance at 450 nm (OD450) was measured on the first day. The CCK-8 working solution was prepared by mixing 1 mL of CCK-8 stock solution (Beyotime Biotechnology Research Institute) with 9 mL of serum-free culture medium. All operations were performed under dark conditions. The culture medium in the wells to be measured was discarded, and 100 μL of the CCK-8 working solution was added to each well. The plate was then incubated in a 37°C cell incubator in the dark for 1 hour. After incubation, the absorbance at 450 nm was measured for each well using a multimode microplate reader. This measurement was repeated every 24 hours for a total of five time points. The OD450 value of blank wells was subtracted from that of the experimental wells. After measurement and calculation, cell growth curves were plotted using Prism software.

2.8. Wound healing assay

Cells were digested and counted. A total of 8×10^5 cells per well were seeded into 6-well plates, with three wells for each cell type. After 24 hours, when the cells had formed a confluent monolayer covering the entire bottom of the wells, the culture medium was replaced with serum-free medium, and the cells were starved for 2 hours. Subsequently, a straight line was quickly and uniformly scratched in the center of each well using a 200 μL pipette tip held vertically. The wells were gently washed twice with PBS (Wuhan Servicebio Technology Co., Ltd.), and then 2 mL of serum-free medium was added. The 6-well plate was placed under an inverted microscope, and images of the same position in each well were captured at 24 hours and 48 hours using a low-power lens (4×). ImageJ software was used to quantify the remaining wound area at 24 hours and 48 hours relative to the initial area at 0 hours. After measurement and calculation, cell migration rate graphs were plotted using Prism software.

2.9. Data Analysis

IBM SPSS 21 software was used for statistical analysis of all data. For data that exhibited homogeneity of variance and normal distribution, a *t*-test was applied. For data that did not meet these assumptions, a nonparametric test was used. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. SP1 is a possible pro-inflammatory gene in pancreatic cancer

The expression of SP1 in pancreatic cancer was discovered from a website for online bioinformatics analysis,

Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/), based on data from The Cancer Genome Atlas (TCGA). The results showed that SP1 expression was low in normal pancreatic tissues but relatively high in pancreatic cancer tissues (**Figure 1A**). KaplanMeier (KM) survival analysis indicated that patients with high SP1 expression tended to have shorter overall survival and recurrencefree survival compared with those with low SP1 expression (**Figure 1B & 1C**). To further validate SP1 expression levels in tumor and normal pancreatic tissues, RTqPCR and Western blot assays were performed (**Figure 1D & 1E**). The results consistently showed that SP1 was upregulated in tumor specimens. Taken together, these findings suggest that SP1 may serve as a potential protumorigenic gene in pancreatic cancer.

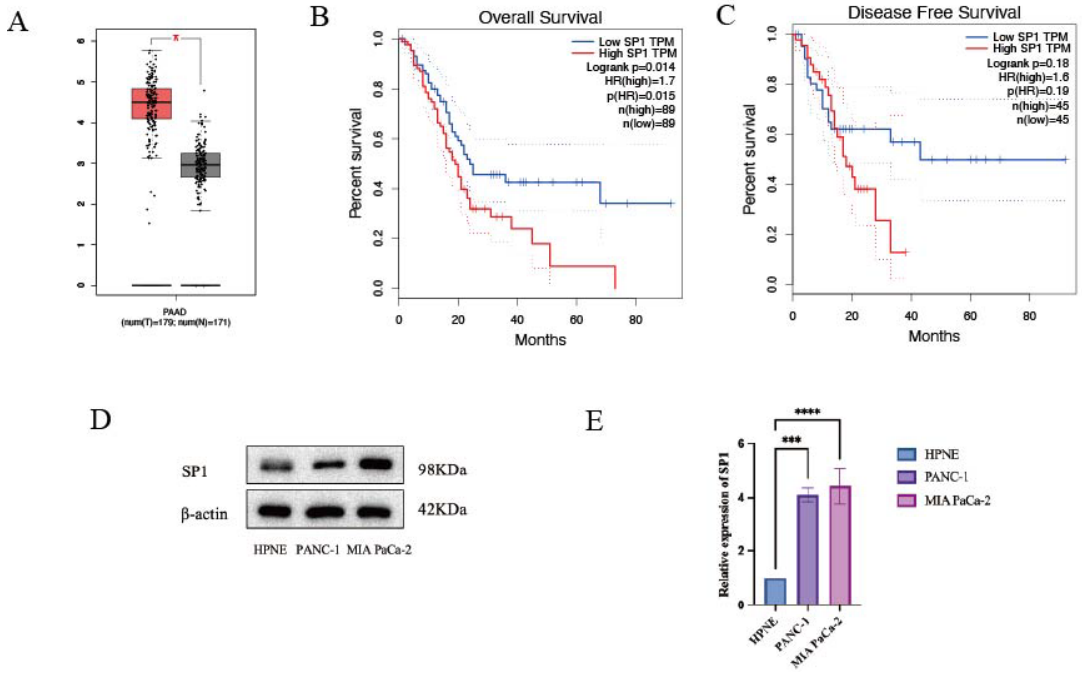

Figure 1. SP1 is highly expressed in pancreatic cancer and acts as a possible pro-tumorigenic gene. (A) SP1 is highly expressed in pancreatic cancer (GEPIA). (B) Pancreatic cancer patients with low SP1 expression have longer overall survival (GEPIA). (C) Pancreatic cancer patients with low SP1 expression have longer disease-free survival (GEPIA). (D) Western blot analysis of SP1 expression in normal pancreatic tissues and pancreatic cancer tissues. (E) RT-qPCR analysis of SP1 expression in normal pancreatic tissues and pancreatic cancer tissues. The above data are presented as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$

To further study the function of SP1 in pancreatic cancer, we transfected SP1 siRNA into MIA PaCa - 2 and PANC - 1 cell lines.

3.2. Knockdown of SP1 suppresses the proliferation and migration of pancreatic cancer cells

As a transcription factor, SP1 has been reported to play a significant role in the occurrence and development of various tumors. To explore the potential function of SP1 in pancreatic cancer, CCK-8 and wound healing assays were performed to evaluate the effects of SP1 knockdown on the proliferation and migration of pancreatic cancer cells. Following SP1 knockdown, the proliferative capacity of pancreatic cancer cells was reduced, according to the CCK-8 assay results (**Figure 2A**). The wound healing assay results indicated that the migratory capacity of pancreatic cancer cells was also decreased after SP1 knockdown (**Figure 2B**), and

subsequent quantitative analysis further supported that cell migration was reduced (**Figure 2C**). According to these findings, SP1 might be a tumor suppressor gene in pancreatic cancer.

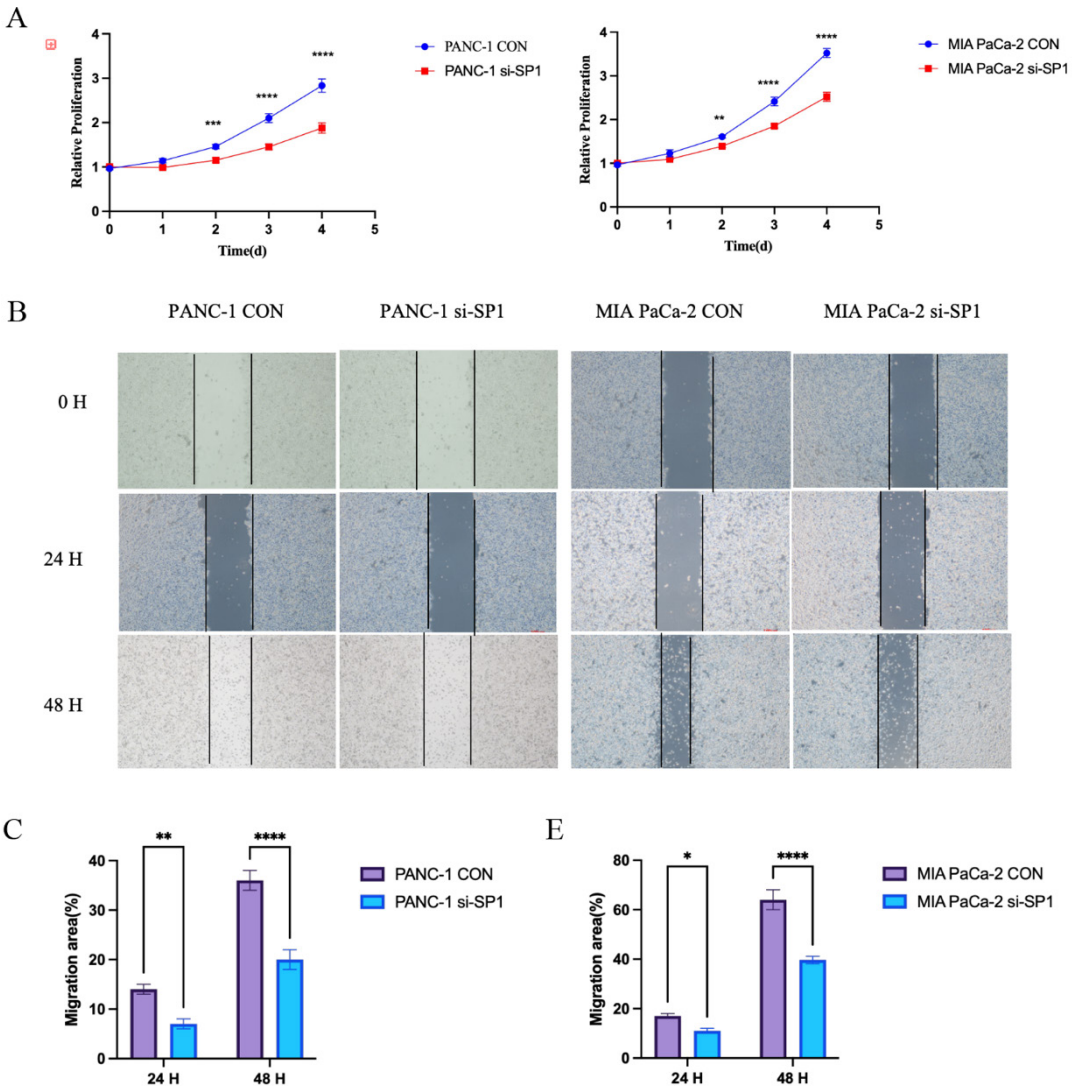

Figure 2. SP1 knockdown prevents pancreatic cancer cells from proliferating and migrating.(A) CCK-8 assay analysis of the proliferative capacity of pancreatic cancer cells in the SP1 knockdown group and the control group. (B) Wound healing assay analysis of the migratory capacity of pancreatic cancer cells in the SP1 knockdown group and the control group. (C) Statistical analysis of the wound healing assay results. (For data with homogeneity of variance and normal distribution, a *t*-test was employed; for data that did not match these requirements, a nonparametric test was applied. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. A *P*-value < 0.05 was considered statistically significant.)

To further study the mechanism of SP1 in pancreatic cancer, we predicted its potential transcription factors using the JASPAR website and found that FOXA3 may be a transcription factor of SP1. The study transfected FOXA3 oe - RNA into MIA PaCa - 2 and PANC - 1 cell lines.

3.3. Overexpression of FOXA3 inhibits SP1 expression

To study the regulatory effect of FOXA3 on SP1, we performed Western blot analysis on MIA PaCa - 2 and

PANC - 1 cells overexpressing FOXA3. The results showed that SP1 expression was lowered following FOXA3 overexpression (**Figure 3**), showing that FOXA3 negatively regulates SP1 production and may be a negative regulator of SP1. Western blot examination of SP1 expression following overexpression of FOXA3.

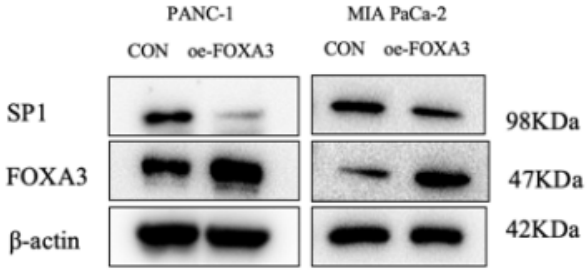

Figure 3. FOXA3 inhibits the expression of SP1.

4. Conclusion

This study shows that SP1 promotes proliferation and migration in pancreatic cancer and, for the first time, reveals that FOXA3 negatively regulates SP1 expression, thereby expanding the transcriptional regulatory network in pancreatic cancer. This axis is useful as a potential therapeutic target and as a biomarker. Compared with previous studies, this work supports the prevailing view of SP1 as a pro-tumorigenic factor and complements the upstream regulatory mechanism, yet lacks molecular evidence of direct binding of FOXA3 to the SP1 promoter as well as in vivo validation. Future research should enhance the chain of evidence through ChIP assays, animal models, and clinical cohorts, as well as investigate combination therapeutic techniques targeting this axis.

Disclosure statement

The author declares no conflict of interest.

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