Cancer Innovation

ORIGINAL ARTICLE OPEN ACCESS

SKIL Promotes Pancreatic Cancer Metastasis by Inhibiting *TSPYL2* to Activate the TGF-β Pathway

Chenxi Wang[1] | Weiwei Song[1] | Yixuan Zhang[2] | Hongming Deng[1] | Zixiang Zhou[3] | Jing Zhu[4] | Xiaobing Wang[1]

[1]State Key Laboratory of Molecular Oncology, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China | [2]NMPA Key Laboratory for Monitoring and Evaluation of Cosmetics, Shanghai Innovation R&D, Testing and Evaluation Technical Service Platform of Cosmetics(22DZ2292100), Shanghai, China | [3]Department of Molecular Biology, Princeton University, Princeton, New Jersey, USA | [4]College of Nursing and Health Innovation, The University of Texas Arlington, Arlington, Texas, USA

Correspondence: Xiaobing Wang (wangxb@cicams.ac.cn)

Received: 16 February 2025 | **Revised:** 24 March 2025 | **Accepted:** 7 April 2025

Funding: This project was supported by the National Natural Science Foundation Fund (Grant Nos. 82372696, 82172988), the Chinese Academy of Medical Sciences (CAMS) Innovation Fund for Medical Sciences (CIFMS) (Grant No. 2021-I2M-1-014), and the Cooperation Fund of CHCAMS and SZCH (Grant No. CFA202201006).

Keywords: epithelial–mesenchymal transition | pancreatic adenocarcinoma | SKIL | TGF-β pathway | TSPYL2

ABSTRACT

Background: Pancreatic adenocarcinoma (PAAD) represents a highly fatal form of cancer. The 5-year survival rate for patients with this disease is only around 10%. A significant hurdle in its management is the absence of characteristic early-stage symptoms. As a result, a large majority of pancreatic cancer patients are diagnosed when the disease has reached an advanced stage or has metastasized. Consequently, taking measures to suppress the occurrence of metastasis in pancreatic cancer can bring about a substantial improvement in patients' survival rates and overall prognosis. *SKIL*, known to promote cancer progression, is implicated in cell proliferation, epithelial–mesenchymal transition (EMT), and metastasis, but its specific function in pancreatic cancer remains unclear.

Methods: We investigated the effects of *SKIL* on the proliferation, apoptosis, and metastasis of pancreatic cancer cells. Through ChIP-seq, we identified the *SKIL* downstream target gene and further explored the mechanism by which *SKIL* regulates the metastasis of pancreatic cancer cells through functional experiments and Western blot.

Results: A high level of *SKIL* expression is associated with an unfavorable prognosis in PAAD; it promotes cell migration and EMT. Through ChIP-seq analysis, we identified that *SKIL* inhibits *TSPYL2*, a nuclear protein regulating the TGF-β pathway by binding to the *TGFB1* promoter. Further studies carried out by us confirmed that *SKIL* modulates the TGF-β pathway via *TSPYL2*, facilitating EMT and metastasis in pancreatic cancer cells, independent of Smad4.

Conclusions: These findings reveal a novel regulatory mechanism involving *SKIL*, *TSPYL2*, and the TGF-β pathway, offering new therapeutic targets for PAAD.

Abbreviations: ChIP, chromatin immunoprecipitation; EMT, epithelial–mesenchymal transition; PAAD, pancreatic adenocarcinoma; *SKIL*, Ski-like protein; TCGA, The Cancer Genome Atlas; TGF-β, transforming growth factor-beta; *TSPYL2*, testis-specific Y-like protein 2.

Chenxi Wang, Weiwei Song, and Yixuan Zhang contributed equally to this work and shared the first authorship.

1 | Introduction

Pancreatic adenocarcinoma (PAAD), which is regarded as a particularly disheartening malignancy, typically shows a 5-year survival rate that is about 10%. It became the 6th major factor resulting in cancer-associated deaths in 2022, representing 5% of the total cancer fatalities worldwide [1]. Localization of pancreatic cancers typically presents no symptoms at the early stage, resulting in over 80% of patients being diagnosed at an advanced or metastatic stage. Consequently, surgical intervention becomes impossible. Owing to inherent chemo resistance and immune resistance, treatment approaches like combined chemotherapy, molecular-targeted medications, and immune checkpoint inhibitors also yield limited effectiveness [2]. Given the comparatively intricate mechanism governing metastasis, at present, there are still no efficient predictive biomarkers or targeted inhibitory strategies for the invasion and metastasis of PAAD. As a result, exploring the crucial molecular mechanisms of proliferation, invasion, and metastasis has become an immediate necessity to boost the treatment effectiveness of PAAD. This is of great practical significance for improving the prognosis of patients with PAAD.

SKIL, also known as Ski-like protein, which belongs to the Ski oncoprotein family, has been found to be involved in the development of various cancer types [3–6]. Earlier research has shown that *SKIL* might play a role in crucial processes. including cell proliferation, epithelial–mesenchymal transition (EMT), and metastasis [7]. EMT is essential for cancer metastasis, as it enables epithelial cells to acquire mesenchymal characteristics, enhancing their motility and invasiveness [8]. Emerging evidence increasingly indicates that *SKIL* may serve as a pivotal regulator of EMT in oncogenesis by interacting with key signaling pathways. Notably, NFAT and *SKIL*-encoded protein SnoN have been shown to cooperatively mediate TGF-β-triggered EMT progression in metastatic breast cancer cell models [9]. Surprisingly, xenotransplantation of breast cancer cells with low SnoN expression led to heightened metastasis to the bone and lung, while tumor development was notably stunted [6]. However, its specific function in pancreatic cancer remains unclear.

The TGF-β signaling pathway is a well-established promoter of EMT and cancer metastasis [10]. In PAAD, aberrant TGF-β signaling correlates with disease progression and poor clinical outcomes, indicating that it could serve as a therapeutic target [11]. Some studies have demonstrated that SnoN binds to Smad4, thereby disrupting the formation of the Smad complexes and subsequently suppressing the TGF-β signaling pathway [12]. However, other research has shown that knocking down endogenous *SKIL* in Mv1Lu cells inhibits TGF-β-induced transcription, but this does not occur in HeLa or HaCaT cells [13]. This suggests that *SKIL* exerts different regulatory effects on the TGF-β pathway, which may depend on the specific cellular context. Smad4 is frequently lost in most pancreatic cancers, and the regulatory role of *SKIL* on TGF-β in both wild-type and Smad4-deficient pancreatic cancer cells remains to be further elucidated.

In addition, *TSPYL2* (testis-specific Y-like protein 2), alternatively termed cell division antigen-1 (CDA1), a nuclear protein, was initially identified as a potential target of TGF-β1 [14]. A previous study has reported that overexpression of *TSPYL2* inhibits HeLa cell growth and colony formation [15]. *TSPYL2* shows reduced expression in mouse primary tumors and human tumors, while its overexpression in human lung cancer and breast cancer cell lines suppresses cellular growth and migration potential [16]. Additionally, *TSPYL2* exerts tumor-suppressive effects in thyroid cancer by participating in interactions with SIRT1 and inhibiting the SIRT1/AKT pathway [17]. These findings suggest a broad role of *TSPYL2* as a tumor suppressor in various cancers, though its role in PAAD is not well characterized [18].

In this study, we demonstrate that *SKIL* is overexpressed in PAAD and correlates strongly with a poor prognosis. High expression of *SKIL* promotes pancreatic cancer cell proliferation by inhibiting apoptosis, while simultaneously enhancing the migration and invasion of cells induced by EMT. Mechanistically, *SKIL* facilitates TGF-β signaling by downregulating *TSPYL2*, thereby further promoting EMT and tumor metastasis. In summary, this study presents a comprehensive investigation into the role of *SKIL* in PAAD, highlighting its value as a potential metastasis biomarker.

2 | Materials and Methods

2.1 | Cell Lines and Transfection

The pancreatic cancer cell lines BxPC-3 and SW1990 were procured from the American Type Culture Collection (ATCC). Meanwhile, the JF305 cell line and the normal pancreatic epithelial cell line CCC-HPE-2 were bought from the National Infrastructure of Cell Line Resource (Beijing, China). These cells were then cultured in Dulbecco's Modified Eagle's Medium (DMEM). The medium was supplemented with 10% fetal bovine serum (FBS) and 1% of an antibiotic solution consisting of 100 U/mL penicillin and 100 mg/mL streptomycin. All cultures were maintained under standardized incubation parameters (37°C, 5% CO_2/95% air mixture) within a humidity-regulated tri-gas incubator system. All experimental procedures were conducted using cells that had been verified to be mycoplasma-free.

Cell transfection to knock down *SKIL* and specific small interfering RNAs (siRNAs) targeting *SKIL* were transfected into BxPC-3 and SW1990 cells using transfection reagent Lipofectamine RNAiMAX (Invitrogen/Thermo Fisher Scientific, Cat# 13778030) according to the manufacturer's instructions. Knockdown efficiency was validated by Western blot and quantitative real-time PCR (RT-qPCR). Similar methods were applied for *TSPYL2* knockdown experiments in BxPC-3 and SW1990 cells. *TSPYL2* overexpression plasmids were obtained from miaolingbio (Wuhan, China). Lipofectamine 3000 (L3000001; Thermo Fisher Scientific) was used for the transfection of the *TSPYL2* overexpression plasmid.

2.2 | Viral Production and Transduction

For lentivirus generation, 2.1 μg of the *SKIL* lentiviral plasmid (miaoling, Wuhan, China) was cotransfected with 1.5 μg of the

psPAX2 plasmid and 0.6 μg of the pMD2.G plasmid. The transfection process was carried out in HEK293T cells using the Neofect™ DNA transfection reagent (TF20121201; Neofect, Beijing, China) at a concentration of 1 μL/mL for the purpose of packaging. 72 h after transfection, the viruses were collected. The lentivirus-containing medium was gathered, pooled, and clarified via centrifugation at 300 g for 10 min at 4°C. JF305 and SW1990 cells were then transduced with the lentivirus. For stable infection selection, the cells were cultured in 2 μg/mL puromycin (Life Technologies, Waltham, MA, USA) for 2 days. RT-qPCR and Western blot analyses were used to confirm the selection.

2.3 | Cell Viability Assay

The viability of cells was evaluated via the Cell Counting Kit-8 (CCK-8) assay (MA0218; Meilunbio, Dalian, China). The experimental procedure strictly adhered to the guidelines provided by the manufacturer. Cells were plated into 96-well plates at a density of 1500 cells per well. Over a period of 6 consecutive days, the CCK-8 reagent was added to the wells every 24 h, strictly following the guidelines provided. Subsequently, the absorbance at a wavelength of 450 nm was measured using a microplate reader (Bio-Rad Laboratories; Hercules, CA, USA). Regarding the colony formation assays, cells were initially seeded at a low density into 6-well plates. After 12 days, colonies were fixed with methanol, stained with crystal violet, and then counted.

2.4 | Apoptosis Analysis

Apoptosis was quantified using an Annexin V-FITC/PI apoptosis detection kit (AD10 ; Dojindo, Kumamoto, Japan) in combination with flow cytometric analysis. In brief, cells were collected, washed with PBS, and the pre-prepared 1 × Annexin V Binding Solution was added to prepare a cell suspension with a final concentration of 1×10^6 cells/mL; the volume of the cell suspension had to be no less than 500 μL. 100 μL of the above cell suspension was added to a new flow tube. Then, 5 μL of Annexin V, FITC conjugate, and 5 μL of PI Solution were added. Incubation of the cells was performed in the absence of light. After that, the apoptosis results were detected using a flow cytometer (Beckman Coulter), and the data were analyzed and plotted using FlowJo v10 (FlowJo LLC) software.

2.5 | Transwell Assay

12 h before the experiment, the serum was removed from the culture medium, and the abilities of invasion and metastasis of the cells were assessed using transwell inserts with an 8-μm pore size (Corning Inc, Corning, NY, USA). With the migration assay initiated by seeding cells in serum-free medium in the upper chamber and adding FBS-supplemented medium below, after 24 h of incubation, the cells were fixed, stained, and counted. The cell invasion assays were conducted using the same procedure as that described above, with the exception that 70 μL of Matrigel (diluted 1:8; Corning Inc., Corning, NY, USA) was applied to pre-coat the upper chamber of the wells.

2.6 | RNA Extraction and Quantitative Real-Time PCR

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), and cDNA was prepared from RNA (500 ng) using the PrimeScript RT Reagent Kit (TaKaRa; Tokyo, Japan) according to the manufacturer's instructions. Quantitative real-time PCR was conducted using the SYBR® Premix Ex Taq™ (TaKaRa) on an ABI V7 system (Applied Biosystems; Indianapolis, IN, USA).

2.7 | Western Blot Analysis

Total protein was extracted from the cells using a lysis buffer containing 1 M Tris-HCl (pH 6.8), 80% glycerol, and 10% SDS. Protein levels were quantified using the BCA protein assay kit (Beyotime Biotech, Nantong, China). Protein samples of equivalent amounts were subjected to SDS-PAGE and were thereafter transposed onto PVDF membranes. The membranes were impeded with 5% nonfat milk and incubated in a 4°C environment throughout the night with primary antibodies against SnoN (Proteintech: 1:1000), CDA1 (Proteintech: 1:1000), E-cadherin (CST: 1:1000), Vimentin (Proteintech: 1:20000), Snai1 (Proteintech: 1:1000), Bax (Abclonal: 1:2000), Bcl-2 (Proteintech: 1:2000), p-Smad2/3 (Affinity: 1:1000), Smad2/3 (Affinity: 1:1000), TGF-β1 (Abcam: 1:5000), and β-actin (ABconal: 1:5000). After washing, the membranes were incubated with HRP-linked secondary antibodies (Zsbio: 1:10,000) and detection was performed using enhanced chemiluminescence.

2.8 | Chromatin Immunoprecipitation Sequencing (ChIP-Seq)

ChIP analysis was conducted using a ChIP assay kit (17-295; Millipore, USA) according to the protocol provided. Cells were treated with formaldehyde for crosslinking, followed by cell lysis and chromatin fragmentation through sonication. An anti-SnoN antibody was used to immunoprecipitate SnoN-bound DNA. Purified DNA was sequenced and analyzed to identify *SKIL*-regulated genes using R version 4.3.3 (Vienna, Austria).

2.9 | The Cancer Genome Atlas (TCGA) Data Analysis

RNA sequencing data along with clinical details for pancreatic cancer patients were retrieved from The Cancer Genome Atlas (TCGA) repository. Kaplan–Meier survival analysis and log-rank tests were used to compare survival curves. Correlation analysis was performed using GEPIA2 [19].

2.10 | Clinical Samples

Samples of 10 pancreatic adenocarcinoma patients were collected from the Cancer Hospital Chinese Academy of

Medical Science. Following approval from the Ethics Committee of the Cancer Hospital Chinese Academy of Medical Sciences (approval number: 21/509-3180), and after obtaining informed consent from all patients, the samples were collected.

2.11 | Statistical Analysis

Each experiment was performed independently at least three times, with the results presented as mean ± standard deviation (SD). Statistical significance was assessed using Student's *t*-test or one-way ANOVA for comparisons involving multiple groups. A p-value < 0.05 was considered statistically significant. Analyses were conducted using GraphPad Prism 10 (GraphPad, La Jolla, CA).

3 | Results

3.1 | *SKIL* Expression Is Upregulated in Pancreatic Cancer and Correlates With a Poor Prognosis

To explore *SKIL* expression across various tumors, we utilized data from the TCGA database, analyzing RNA-sequencing profiles across multiple cancer types. A pan-cancer analysis revealed significantly elevated *SKIL* expression in tumor tissues than in normal tissues (Figure 1a). In PAAD specifically, *SKIL* expression was significantly higher in tumor tissues ($n = 179$) than in adjacent normal tissues ($n = 171$) (Figure 1b). This suggests that *SKIL* is overexpressed in pancreatic cancer. To assess the clinical relevance of *SKIL* expression, we performed Kaplan–Meier survival analyses. Our results showed that high

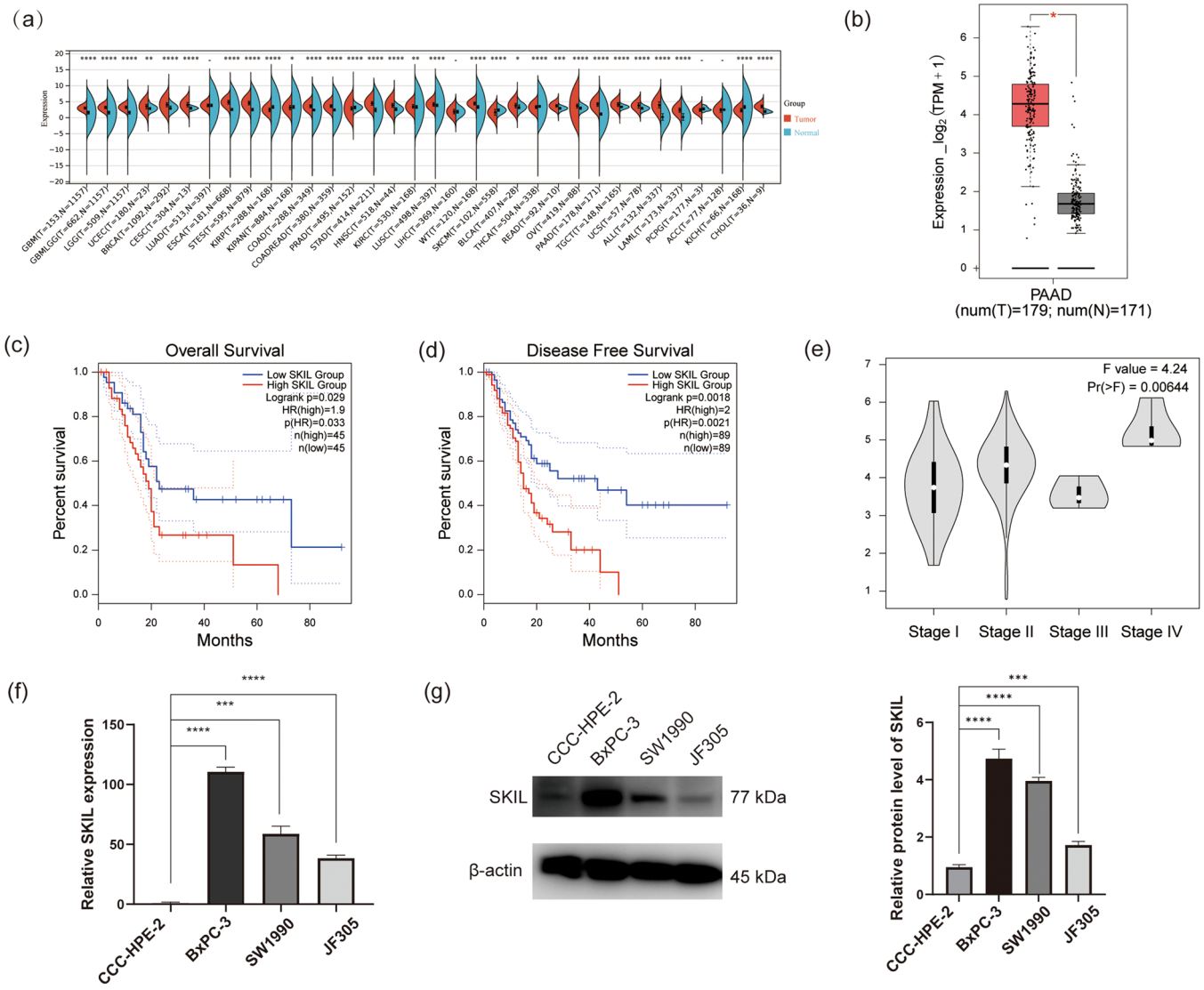

FIGURE 1 | *SKIL* expression and prognostic significance in pancreatic cancer. (a) *SKIL* expression across various cancer types, with higher levels observed in tumor tissues (red) compared to normal tissues (blue). (b) Boxplot compares *SKIL* expression in PAAD tumor tissues ($n = 179$) and adjacent normal tissues ($n = 171$), showing significantly elevated levels in tumor tissues. (c) Kaplan–Meier survival analysis indicates worse overall survival in PAAD patients with high *SKIL* expression. (d) Kaplan–Meier analysis demonstrates significantly reduced disease-free survival in PAAD patients with elevated *SKIL* expression. (e) Violin plot reveals progressively higher *SKIL* expression with advancing PAAD disease stages (I–IV). (f, g) RT-qPCR and Western blot analysis show increased *SKIL* expression in pancreatic cancer cell lines (BxPC-3, SW1990, JF305) compared to a normal pancreatic epithelial cell line (CCC-HPE-2). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

SKIL expression is associated with worse overall survival (OS) in PAAD patients (Figure 1c). Furthermore, disease-free survival (DFS) analysis revealed that patients with elevated *SKIL* expression experienced shorter DFS, highlighting a correlation between high *SKIL* levels and poor clinical outcomes (Figure 1d). Analysis of *SKIL* expression at different disease stages demonstrated a positive association between *SKIL* levels and disease progression, with the highest expression observed in stage IV tumors (Figure 1e). These findings suggest that *SKIL* expression increases as pancreatic cancer advances, potentially contributing to tumor progression.

Next, we examined *SKIL* expression in various pancreatic cancer cell lines and a normal pancreatic epithelial cell line (CCC-HPE-2). RT-qPCR and Western blot analysis indicated significantly lower *SKIL* expression in normal pancreatic epithelial cells, while *SKIL* was markedly elevated in pancreatic cancer cell lines such as BxPC-3, SW1990, and JF305 (Figure 1f,g). These data collectively suggest that *SKIL* is consistently upregulated in both PAAD tissues and cell lines, supporting its role as a potential biomarker for pancreatic cancer progression and poor prognosis.

3.2 | *SKIL* Facilitates Pancreatic Cancer Cell Proliferation by Suppressing Apoptosis

To explore the role of *SKIL* in promoting pancreatic cancer cell growth, we performed knockdown and overexpression experiments in different pancreatic cancer cell lines. Western blot and Rt-qPCR confirmed effective *SKIL* knockdown in BxPC-3 and SW1990 cells (Figure 2a) and overexpression in JF305 and SW1990 cells (Figure 2b). CCK8 assays demonstrated that *SKIL* knockdown significantly reduced cell viability, whereas *SKIL* overexpression enhanced it (Figure 2c,d). Colony formation assays further supported these findings, showing that *SKIL* knockdown resulted in fewer colonies in BxPC-3 and SW1990 cells (Figure 2e), while *SKIL* overexpression increased colony formation in JF305 and SW1990 cells (Figure 2f). These results suggest that *SKIL* plays a pivotal role in promoting pancreatic cancer cell proliferation.

We also explored the mechanism of *SKIL*'s effect on apoptosis. Flow cytometry revealed that *SKIL* knockdown significantly increased apoptosis in BxPC-3 cells (Figure 2g), which was further confirmed by the Western blot showing increased Bax (pro-apoptotic protein) and decreased Bcl-2 (anti-apoptotic protein) levels (Figure 2h). Together, these findings indicate that *SKIL* promotes pancreatic cancer cell growth by inhibiting apoptosis.

3.3 | *SKIL* Enhances Migration and Invasion in PAAD, Also Triggering EMT

To investigate whether *SKIL* contributes to the metastatic potential of PAAD cells, we performed migration and invasion assays. Transwell assays demonstrated that *SKIL* knockdown significantly reduced migration and invasion in BxPC-3 and SW1990 cells (Figure 3a,b), while *SKIL* overexpression enhanced these abilities in JF305 and SW1990 cells (Figure 3c,d). Wound healing assays further confirmed *SKIL*'s role in promoting cell migration, as *SKIL* knockdown reduced wound closure rates, whereas overexpression increased them (Figure 3e,f).

Given that EMT holds a crucial and central position in the processes of tumor invasion and metastasis, we examined EMT marker expression following *SKIL* knockdown or overexpression. Western blot analysis showed that *SKIL* knockdown increased the epithelial marker E-cadherin and decreased the mesenchymal markers vimentin and Snai1, indicating an inhibition of EMT (Figure 3g). In contrast, *SKIL* overexpression led to reduced E-cadherin and increased Vimentin and Snai1 expression, consistent with EMT induction (Figure 3h). These outcomes imply that *SKIL* expedites the migratory and invasive capabilities of PAAD via the instigation of the EMT.

3.4 | *SKIL* Regulates the Transcriptional Repression of *TSPYL2*

To elucidate the mechanisms through which *SKIL* promotes PAAD cell migration, we performed chromatin immunoprecipitation sequencing (ChIP-seq) to identify *SKIL*-regulated genes. 5085 regions were identified in the targets (peak position in up 5000 bp) through ChIP-seq (Figure 4a). Then, we conducted prognostic and correlative analyses, ultimately identifying *TSPYL2* as a pivotal candidate gene (Figure 4b). *TSPYL2* shows downregulated expression in pancreatic cancer and is significantly associated with unfavorable prognosis in pancreatic cancer (Figure 4c–e). Notably, analysis of the TCGA data shows a significant negative correlation between *TSPYL2* and *SKIL* expression in pancreatic cancer (Figure 4f). Moreover, we collected tumor tissues from 10 pancreatic cancer patients for immunohistochemical staining of *SKIL* and *TSPYL2* proteins. The results showed that there was a significant negative correlation between the expression levels of *SKIL* and *TSPYL2* proteins (Figure 4g,h). Further validation via Western blot established the regulatory effect of *SKIL* in *TSPYL2*, where knockdown of *SKIL* results in elevated expression of *TSPYL2*, whereas overexpression of *SKIL* suppressed *TSPYL2* expression (Figure 4i–k).

3.5 | *SKIL* Promote Pancreatic Cancer Cell Migration Through the Downregulation of *TSPYL2*

TSPYL2 plays tumor-suppressive roles in various cancers [16, 20–22]. Many investigations have demonstrated that *TSPYL2* exerts an inhibitory effect on the activity of the cyclin B-CDK1 complex [21], and high expression of *TSPYL2* suppresses the proliferation and migration of lung cancer cells [16]. However, the role of *TSPYL2* in pancreatic cancer remains unclear. To investigate whether *TSPYL2* plays a role in pancreatic cancer metastasis, we overexpressed and knocked down *TSPYL2* in BxPC-3 and SW1990 cells (Figure 5a,b). Transwell assays elucidated that the silencing of *TSPYL2* conspicuously increased the migratory capacity of PAAD cells, while overexpression inhibited migration (Figure 5c,d). Western blot experiments revealed that *TSPYL2*

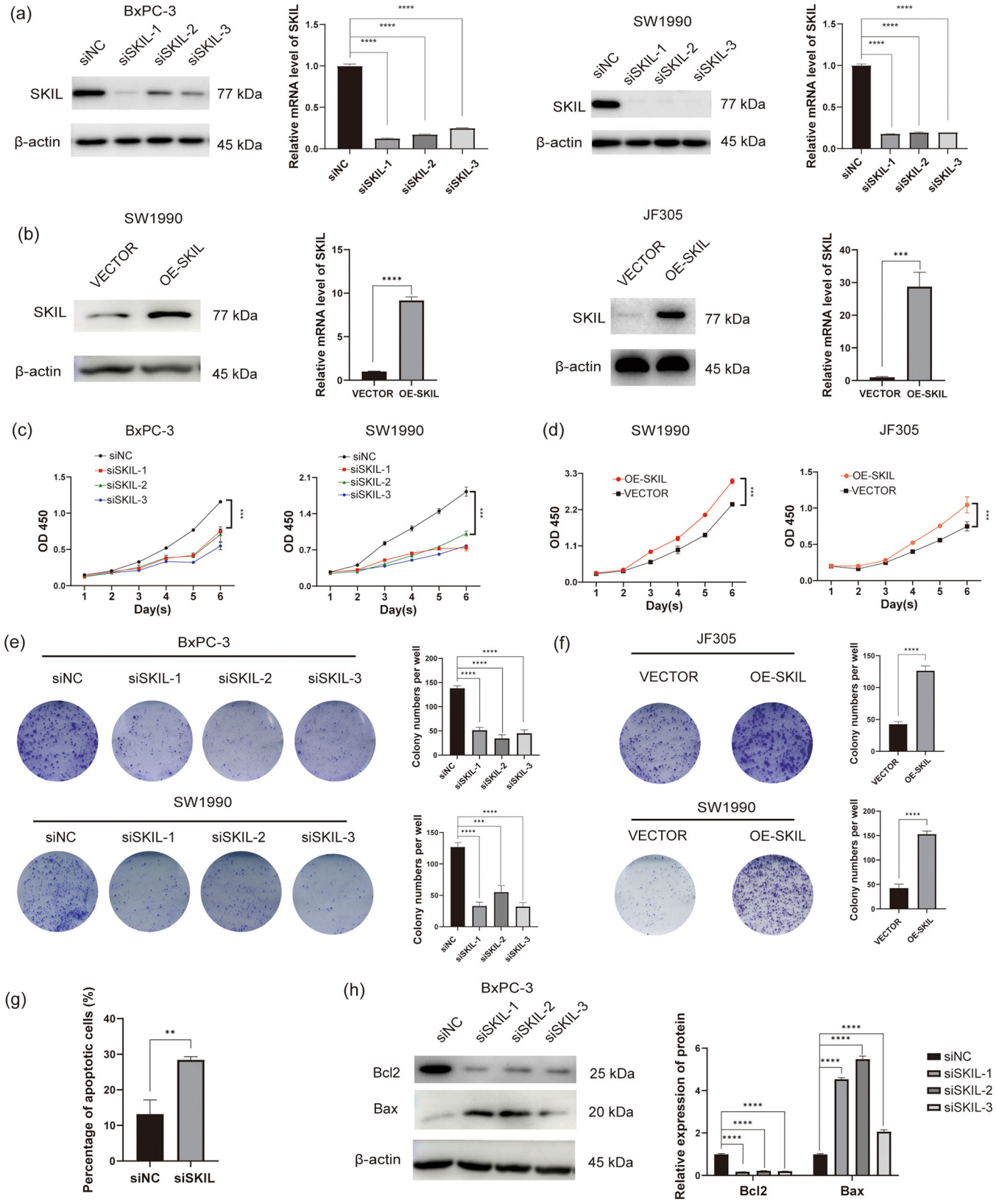

FIGURE 2 | Manipulation of *SKIL* expression in pancreatic cancer cell lines and its impact on cell growth. (a) Western blot and RT-qPCR confirm *SKIL* knockdown in BxPC-3 and SW1990 cells following siRNA treatment. (b) Western blot and RT-qPCR confirm *SKIL* overexpression in SW1990 and JF305 cells. (c, d) CCK-8 assays show decreased cell viability in BxPC-3 and SW1990 cells following *SKIL* knockdown and increased viability in SW1990 and JF305 cells with *SKIL* overexpression. (e, f) Colony formation assays demonstrate reduced colony formation in BxPC-3 and SW1990 cells with *SKIL* knockdown and enhanced colony formation in SW1990 and JF305 cells with *SKIL* overexpression (g, h) Flow cytometry and Western blot analysis show increased apoptosis in BxPC-3 cells with *SKIL* knockdown and altered expression of apoptotic regulators (Bax, Bcl-2). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

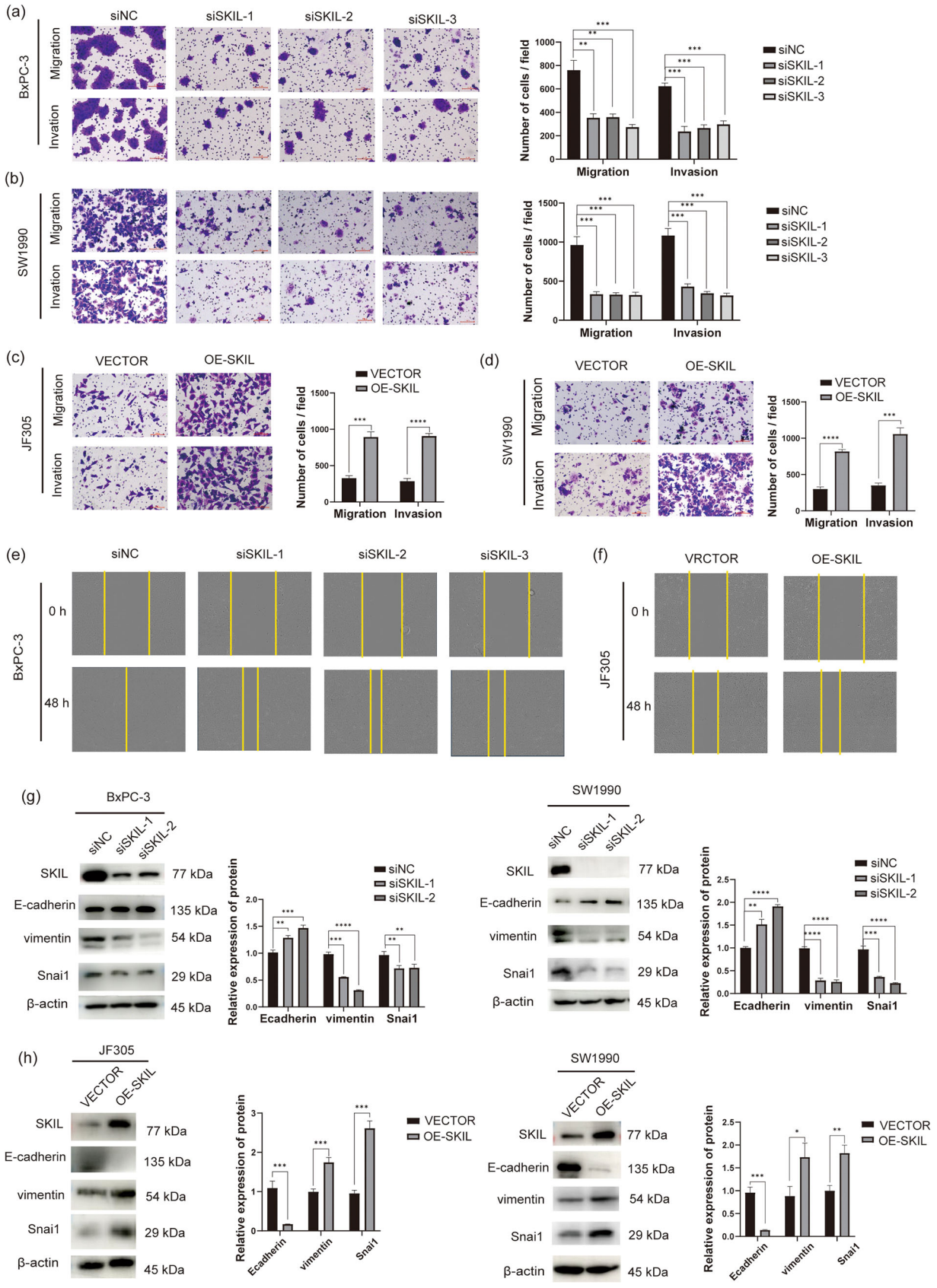

FIGURE 3 | *SKIL* regulates cell migration, invasion, and EMT in pancreatic cancer. (a, b) Transwell assays show reduced migration and invasion in both BxPC-3 and SW1990 cells following *SKIL* knockdown. (c, d) Transwell assays demonstrate enhanced migration and invasion in SW1990 and JF305 cells with *SKIL* overexpression. (e, f) Wound healing assays show slower wound closure in BxPC-3 cells with *SKIL* knockdown and faster closure in JF305 cells with *SKIL* overexpression. (g, h) Western blot assay shows an increase in the expression of E-cadherin and a reduction in the expressions of vimentin and Snai1 within BxPC-3 and SW1990 cells with *SKIL* knockdown and reversed effects in JF305 and SW1990 cells with *SKIL* overexpression. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

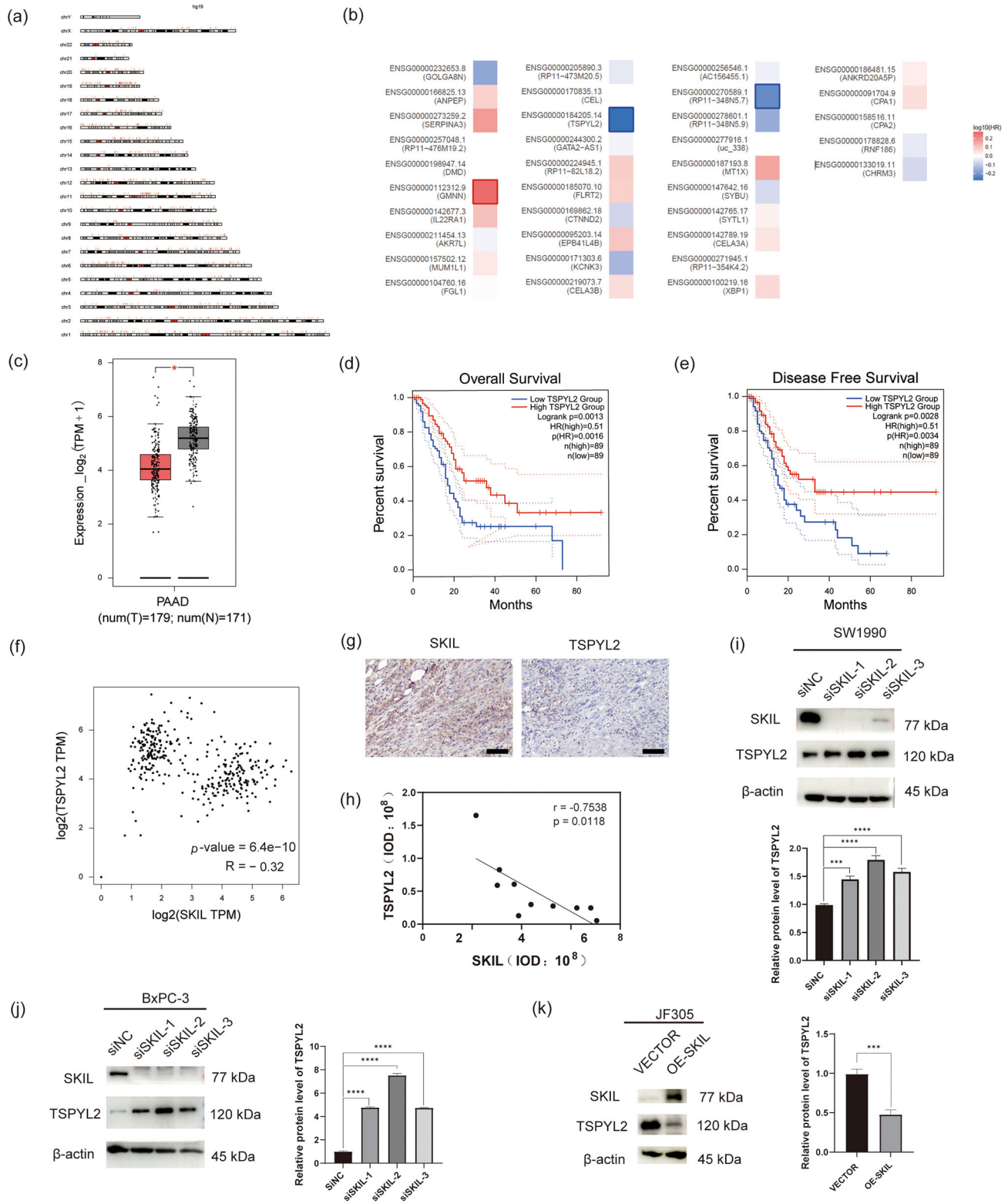

FIGURE 4 | ChIP-seq analysis reveals *TSPYL2* as a downstream target of *SKIL*. (a) Peak map from ChIP-seq analysis illustrates the distribution of *SKIL*-regulated targets across the genome. (b) Heatmap demonstrates the correlation between candidate genes identified by ChIP-seq and pancreatic cancer prognosis. (c) Boxplot compares *TSPYL2* expression in PAAD tumor tissues ($n = 179$) and adjacent normal tissues ($n = 171$), showing significantly downregulated levels in tumor tissues. (d, e) Kaplan–Meier survival analysis indicates poorer overall and disease-free survival in patients with low *TSPYL2* expression. (f) Analysis demonstrates a significant negative correlation in the expression of *SKIL* and *TSPYL2* using the GEPIA database. (g) Representative images of immunohistochemical staining for *SKIL* and *TSPYL2* protein in pancreatic cancer tissues. Scale bars: 100 μm. (h) Correlation of the Integral optical density (IOD) for *SKIL* and *TSPYL2* in pancreatic cancer tissues. (i, j) Western blot shows the effect of *SKIL* knockdown on *TSPYL2* expression in SW1990 and BxPC-3 cells. (k) Western blot shows the effect of *SKIL* overexpression on *TSPYL2* expression in JF305 cells. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

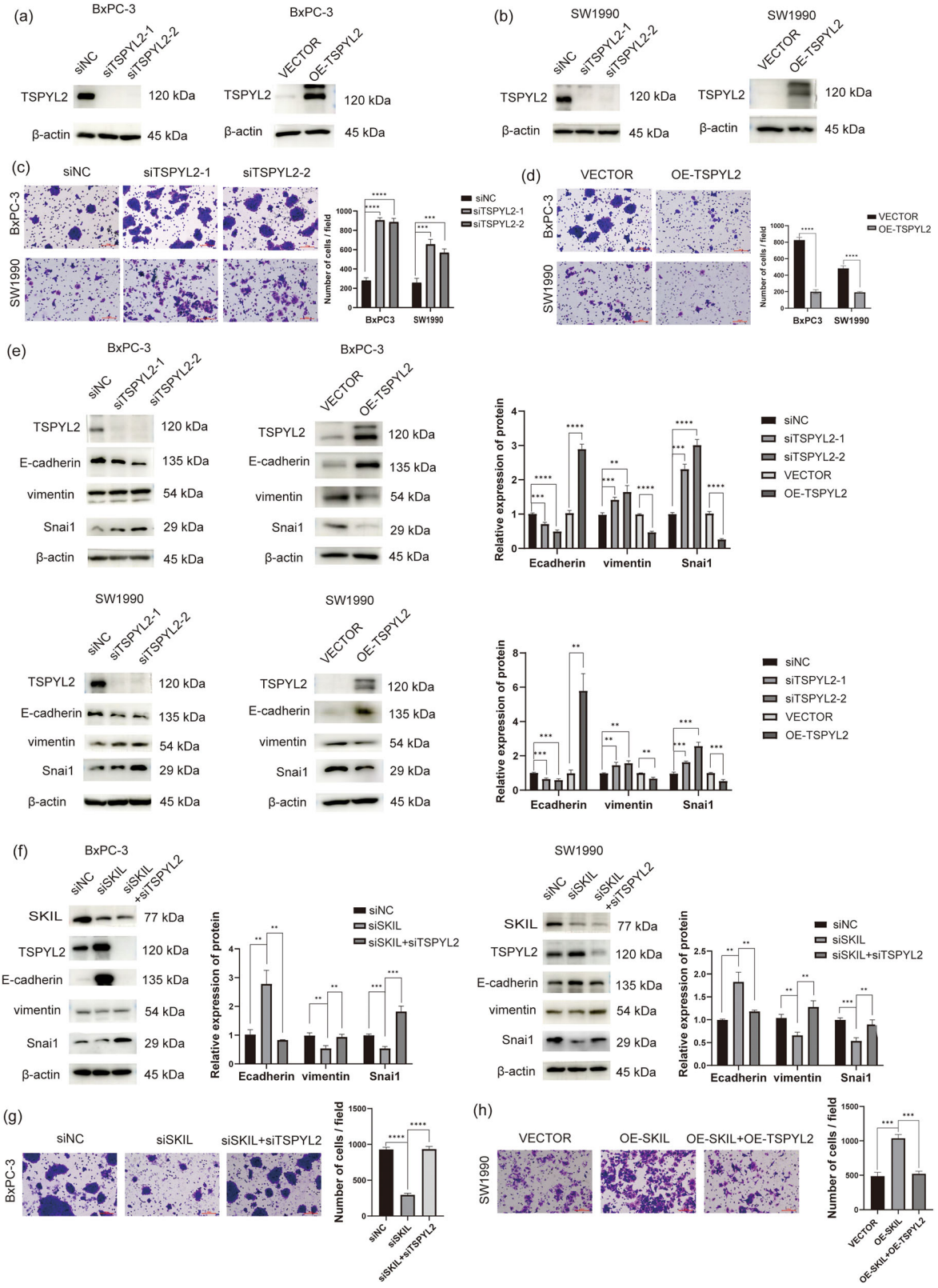

FIGURE 5 | *SKIL* promotes pancreatic cancer cell migration through the downregulation of *TSPYL2*. (a, b) Western blot shows successful knockdown and overexpression of *TSPYL2* in BXPC-3 and SW1990 cells. (c) Transwell assays show elevated migration in BxPC-3 and SW1990 cells with *TSPYL2* knockdown. (d) Transwell assays show reduced migration in BxPC-3 and SW1990 cells with *TSPYL2* overexpression. (e) Western blot analysis shows the expression of major EMT markers (E-cadherin, vimentin, Snai1) following manipulation of *TSPYL2* expression in BxPC-3 and SW1990 cells. (f) Western blot analysis demonstrates that knocking down *TSPYL2* following *SKIL* knockdown reversed major EMT markers in BxPC-3 and SW1990 cells. (g) Transwell assays show enhanced migration in BxPC-3 and SW1990 cells with *TSPYL2* knockdown following *SKIL* knockdown. (h) Transwell assays show reduced migration in BxPC-3 and SW1990 cells, with *TSPYL2* overexpression following *SKIL* overexpression. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

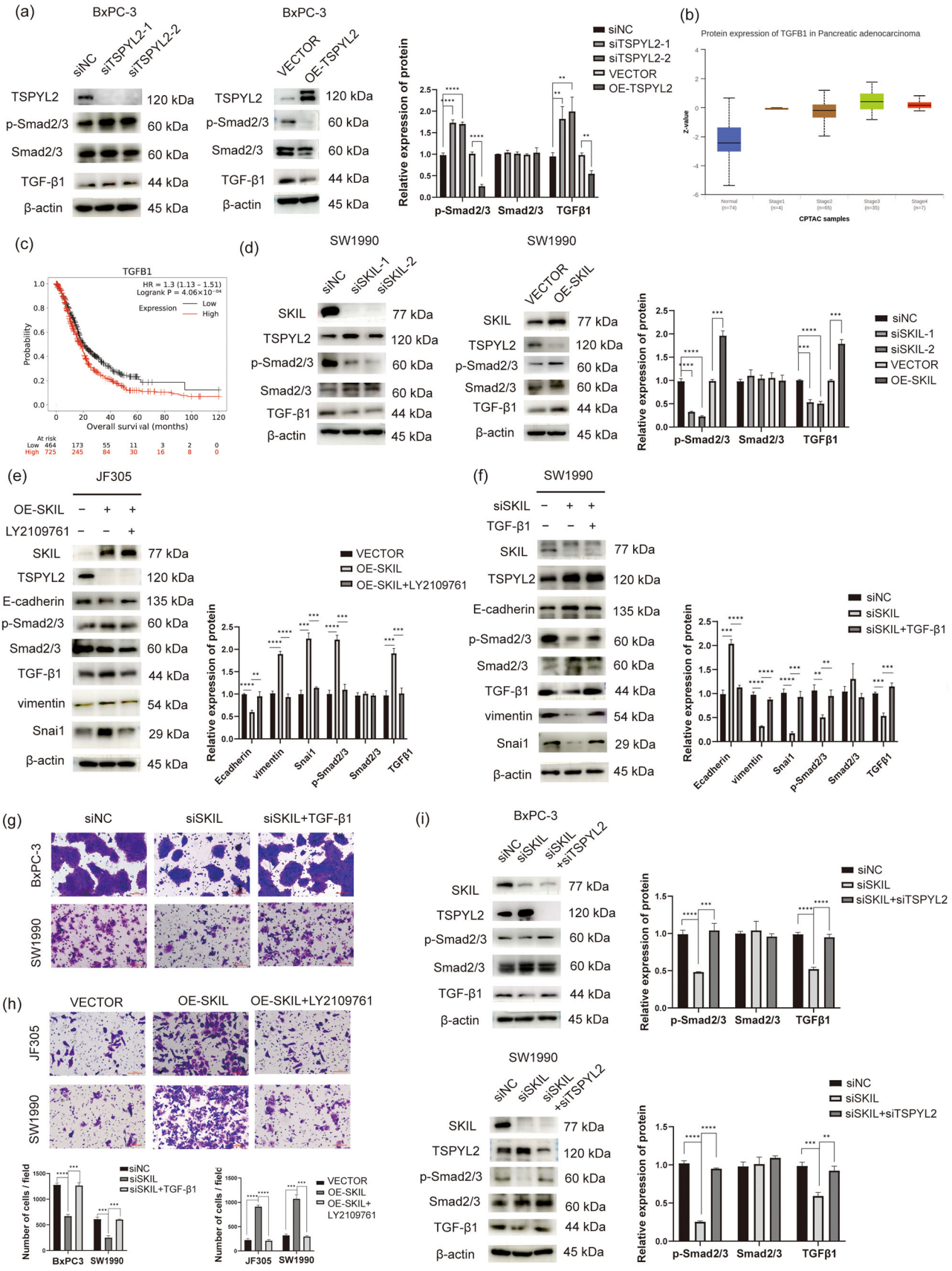

FIGURE 6 | Legend on next page.

knockdown reduced the expression of E-cadherin and increased the expression of Vimentin and Snai1, promoting the EMT process, whereas *TSPYL2* overexpression suppressed EMT (Figure 5e).

Rescue experiments were performed to determine if *SKIL* regulates PAAD migration through *TSPYL2*. Western blot results showed that silencing *TSPYL2* in *SKIL*-knockdown cells reversed the EMT marker changes and restored cell migration (Figure 5f,g). Conversely, overexpression of *TSPYL2* in *SKIL*-overexpressing cells inhibited migration (Figure 5h). These findings demonstrate that *SKIL* promotes PAAD cell migration by downregulating *TSPYL2*, thereby inducing EMT.

3.6 | *SKIL* Induces EMT in PAAD Cells by Downregulating *TSPYL2* and Activating the TGF-β Signaling Pathway

Preceding investigations have implied that *TSPYL2* is capable of binding to the promoter domains of the *TGFB1* gene [23]. To explore whether *TSPYL2* influences the TGF-β pathway in PAAD cells, we performed Western blot analysis, which revealed that *TSPYL2* knockdown activated TGF-β signaling, increasing Smad2/3 phosphorylation and TGF-β1 expression. Conversely, overexpression of *TSPYL2* inhibited TGF-β signaling, reducing Smad2/3 phosphorylation and TGF-β1 levels (Figure 6a, Figure S1a). Meanwhile, The TCGA analysis revealed that TGF-β1 expression significantly increased in advanced pancreatic cancer (Figure 6b). Kaplan–Meier evaluation demonstrated that increased expression of *TGFB1* correlated with lower survival rates in PAAD patients (Figure 6c). To explore the interaction between *SKIL* and the TGF-β pathway, we assessed the impact of *SKIL* knockdown and overexpression on TGF-β signaling in PAAD. Western blot analysis revealed that silencing *SKIL* reduced both Smad2/3 phosphorylation and TGF-β1 expression, whereas *SKIL* overexpression led to an increase in both (Figure 6d, Figure S1b). These findings suggest that elevated *SKIL* expression may promote PAAD progression through the TGF-β pathway.

The TGF-β pathway plays a crucial role in EMT induction, and we further investigated whether *SKIL* mediates EMT in PAAD cells through the regulation of TGF-β signaling. The results showed that treatment of *SKIL*-knockdown cells with recombinant TGF-β1 restored EMT and migration, while treatment with the TGF-β inhibitor LY2109761 in *SKIL*-overexpressing cells reversed EMT induction and reduced migration (Figure 6e–h). These results provide functional evidence that the TGF-β pathway is a critical mediator of *SKIL*-induced EMT.

Additionally, we investigated whether *SKIL* influences TGF-β signaling through the suppression of *TSPYL2*. Western blot results showed that simultaneous knockdown of *TSPYL2* in *SKIL*-silenced cells reversed the reduction in Smad2/3 phosphorylation and TGF-β1 levels caused by *SKIL* knockdown (Figure 6i). These findings confirm that *SKIL* promotes EMT and cell migration by suppressing *TSPYL2*, thereby activating the TGF-β signaling pathway.

4 | Discussion

Pancreatic cancer is a highly malignant tumor characterized by strong invasiveness and poor prognosis [1, 24]. Due to the lack of typical early symptoms, most pancreatic cancer patients are diagnosed at an advanced stage or with metastasis. Therefore, inhibiting the incidence of metastasis in pancreatic cancer can significantly improve patient survival and prognosis [2]. Cancer metastasis is the leading cause of cancer-related deaths, and EMT is a key mechanism of cancer metastasis. EMT transforms epithelial cells into more invasive mesenchymal cells, which is considered a hallmark of pancreatic cancer metastasis [25–27]. Identification of targets related to EMT is an effective strategy to enhance the survival rate of pancreatic cancer patients.

SKIL is an oncogenic and tumor-suppressive molecule with a dual role [6, 28, 29]. At present, its most extensively documented function pertains to the regulation of the TGF-β signaling pathway. The *SKIL*-encoded protein SnoN engages R-Smad (Smad2/3) through its N-terminal domain and co-Smad (Smad4) via the SAND-like region. The competitive binding of SnoN and R-Smad to Smad4 disrupts the assembly of functional heteromeric Smad complexes, thereby impeding the transcriptional activation of TGF-β target genes by Smad complexes [12, 30]. This mechanism underscores *SKIL*'s designation as a negative regulator of the TGF-β pathway. Downregulation of SnoN augments TGF-β-induced EMT in A549 and MDA-MB-231 cell lines [6]. Nonetheless, a burgeoning body of evidence indicates that *SKIL*'s regulatory impact on the TGF-β pathway is multifarious. Walldén et al. [31] propose that SnoN does not dismantle the formation of heteromeric Smad complexes; instead, it fortifies the Smad3–Smad4 complex. In another investigation, knockdown of endogenous *SKIL* in the lung epithelial cell line Mv1Lu curtailed TGF-β-induced transcription, contradicting the characterization of *SKIL* functions as an inhibitory modulator within the TGF-β signaling pathway [13].

FIGURE 6 | *SKIL* induces EMT in PAAD cells by downregulating *TSPYL2* and activating the TGF-β signaling pathway. (a) Western blot shows the effect of *TSPYL2* on the TGF-β pathway. (b) Levels of TGF-β1 protein at different stages of pancreatic cancer progression. (c) Elevated expression of *TGFB1* has been implicated in dismal prognoses within the context of pancreatic cancer. (d) Western blot shows the effect of *SKIL* on the TGF-β pathway. (e) Treatment with TGF-β receptor type I/II inhibitors reverses the activation of EMT and TGF-β-related molecules induced by *SKIL* overexpression. (f) Treatment with TGF-β recombinant protein restores the suppression of EMT- and TGF-β-related molecules caused by *SKIL* knockdown. (g) Treatment with TGF-β recombinant protein restores the impaired cell migration capability caused by *SKIL* knockdown. (h) Treatment with the TGF-β receptor inhibitors reverses the enhanced cell migration capability induced by *SKIL* overexpression. (i) Knockdown of *TSPYL2* following *SKIL* knockdown in BxPC-3 and SW1990 cells restores the inhibition of the TGF-β pathway caused by *SKIL* knockdown. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Overall, these studies suggest that the regulatory effects of *SKIL* on the TGF-β pathway are cell context-dependent, contingent on the specific cellular environment.

In our study, we utilized Smad4 wild-type cells SW1990 and JF305, as well as Smad4-deficient cells BxPC-3, to simulate a broader spectrum of scenarios, aligning more closely with the clinical reality where Smad4 is inactivated in 60% of pancreatic cancers [32]. However, we observed that high expression of *SKIL* promoted pancreatic cancer cell migration and EMT in both Smad4 wild-type and Smad4-deficient cells. This led us to hypothesize that *SKIL*-induced EMT and cell migration in pancreatic cancer cells are independent of the Smad-TGF-β pathway. Given that *SKIL* functions as a transcriptional co-factor [33], we used ChIP-seq to identify *SKIL*'s downstream target genes, discovering that *SKIL* instigates the EMT and metastatic dissemination in pancreatic cancer cells via the downregulation of *TSPYL2* expression. Interestingly, the most extensively studied function of *TSPYL2* remains its regulation of the TGF-β pathway, as *TSPYL2* has been found to directly bind to the promoter region of the *TGFB1* gene [33]. Further investigations confirmed that in pancreatic cancer cells, *SKIL* regulates the TGF-β pathway by modulating *TSPYL2*, independent of Smad4.

However, this study has certain limitations: the regulatory sites of *SKIL* on *TSPYL2* have not yet been confirmed, and the research lacks in vivo experiments to further substantiate the findings. Future studies will address these gaps and provide further validation.

5 | Conclusion

In summary, we have identified a novel mechanism by which *SKIL* regulates the TGF-β pathway and induces EMT and metastasis in pancreatic cancer cells, providing new insights into the relationship among *SKIL*, *TSPYL2*, and the TGF-β pathway, and holds great potential for therapeutic applications. On the one hand, *SKIL* plays a crucial role in promoting the migration and EMT of pancreatic cancer cells, so it is a promising therapeutic target. One potential approach is to develop small-molecule inhibitors that specifically target *SKIL*. These inhibitors can disrupt the binding of *SKIL* to its downstream targets (such as *TSPYL2*), thereby blocking the *SKIL*-induced downregulation of *TSPYL2* and the subsequent activation of the TGF-β pathway. On the other hand, since *SKIL* regulates the TGF-β pathway by modulating *TSPYL2*, restoring the expression of *TSPYL2* may be a therapeutic strategy. Small-molecule activators of *TSPYL2* can be developed to inhibit the TGF-β pathway and further suppress the EMT and metastasis of pancreatic cancer cells.

Author Contributions

Chenxi Wang: data curation (equal), formal analysis (lead), investigation (equal), methodology (equal), writing – original draft (equal). **Weiwei Song:** data curation (equal), methodology (lead), writing – original draft (equal). **Yixuan Zhang:** conceptualization (equal), formal analysis (equal), investigation (equal), methodology (equal), validation (lead). **Hongming Deng:** investigation (equal), methodology (equal). **Zixiang Zhou:** formal analysis (equal). **Jing Zhu:** formal analysis (equal). **Xiaobing Wang:** conceptualization (equal), funding acquisition (lead), project administration (lead), writing – review and editing (lead).

Acknowledgments

The authors have nothing to report.

Ethics Statement

This study was approved by the Ethics Committee of Cancer Hospital Chinese Academy of Medical Sciences (approval number: 21/509-3180).

Consent

All patients provided written informed consent at the time of entry in this study.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. F. Bray, M. Laversanne, H. Sung, et al., "Global Cancer Statistics 2022: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries," *CA: A Cancer Journal for Clinicians* 74, no. 3 (2024): 229–263, https://doi.org/10.3322/caac.21834.

2. J. D. Mizrahi, R. Surana, J. W. Valle, and R. T. Shroff, "Pancreatic Cancer," *Lancet* 395, no. 10242 (2020): 2008–2020, https://doi.org/10.1016/S0140-6736(20)30974-0.

3. D. Hagerstrand, A. Tong, S. E. Schumacher, et al., "Systematic Interrogation of 3q26 Identifies TLOC1 and Skil as Cancer Drivers," *Cancer Discovery* 3, no. 9 (2013): 1044–1057, https://doi.org/10.1158/2159-8290.Cd-12-0592.

4. I. Imoto, A. Pimkhaokham, Y. Fukuda, et al., "Sno Is a Probable Target for Gene Amplification at 3q26 in Squamous-Cell Carcinomas of the Esophagus," *Biochemical and Biophysical Research Communications* 286, no. 3 (2001): 559–565, https://doi.org/10.1006/bbrc.2001.5428.

5. N. Nomura, S. Sasamoto, S. Ishii, T. Date, M. Matsui, and R. Ishizaki, "Isolation of Human cDNA Clones of Ski and the Ski-Related Gene, Sno," *Nucleic Acids Research* 17, no. 14 (1989): 5489–5500, https://doi.org/10.1093/nar/17.14.5489.

6. Q. Zhu, A. R. Krakowski, E. E. Dunham, et al., "Dual Role of SnoN in Mammalian Tumorigenesis," *Molecular and Cellular Biology* 27, no. 1 (2007): 324–339.

7. M. A. Briones-Orta, L. Levy, C. D. Madsen, et al., "Arkadia Regulates Tumor Metastasis by Modulation of the TGF-β Pathway," *Cancer Research* 73, no. 6 (2013): 1800–1810, https://doi.org/10.1158/0008-5472.CAN-12-1916.

8. Y. Huang, W. Hong, and X. Wei, "The Molecular Mechanisms and Therapeutic Strategies of EMT in Tumor Progression and Metastasis," *Journal of Hematology & Oncology* 15, no. 1 (2022): 129, https://doi.org/10.1186/s13045-022-01347-8.

9. S. Sengupta, S. Jana, S. Biswas, P. K. Mandal, and A. Bhattacharyya, "Cooperative Involvement of NFAT and SnoN Mediates Transforming Growth factor-β (TGF-β) Induced EMT in Metastatic Breast Cancer (MDA-MB 231) Cells," *Clinical & Experimental Metastasis* 30, no. 8 (2013): 1019–1031, https://doi.org/10.1007/s10585-013-9600-y.

10. X. Wang, P. J. A. Eichhorn, and J. P. Thiery, "TGF-β, EMT, and Resistance to Anti-Cancer Treatment," *Seminars in Cancer Biology* 97 (2023): 1–11, https://doi.org/10.1016/j.semcancer.2023.10.004.

11. L. Qiang, M. T. Hoffman, L. R. Ali, et al., "Transforming Growth Factor-β Blockade in Pancreatic Cancer Enhances Sensitivity to Combination Chemotherapy," *Gastroenterology* 165, no. 4 (2023): 874–890.e10, https://doi.org/10.1053/j.gastro.2023.05.038.

12. J. W. Wu, A. R. Krawitz, J. Chai, et al., "Structural Mechanism of Smad4 Recognition by the Nuclear Oncoprotein Ski," *Cell* 111, no. 3 (2002): 357–367, https://doi.org/10.1016/s0092-8674(02)01006-1.

13. K. P. Sarker, S. M. Wilson, and S. Bonni, "SnoN Is a Cell Type-Specific Mediator of Transforming Growth Factor-β Responses," *Journal of Biological Chemistry* 280, no. 13 (2005): 13037–13046, https://doi.org/10.1074/jbc.M409367200.

14. L. L. Ozbun, L. You, S. Kiang, J. Angdisen, A. Martinez, and S. B. Jakowlew, "Identification of Differentially Expressed Nucleolar TGF-β1 Target (DENTT) in Human Lung Cancer Cells That Is a New Member of the TSPY/SET/NAP-1 Superfamily," *Genomics* 73, no. 2 (2001): 179–193, https://doi.org/10.1006/geno.2001.6505.

15. Z. Chai, B. Sarcevic, A. Mawson, and B. H. Toh, "SET-Related Cell Division Autoantigen-1 (CDA1) Arrests Cell Growth," *Journal of Biological Chemistry* 276, no. 36 (2001): 33665–33674, https://doi.org/10.1074/jbc.M007681200.

16. L. E. Kandalaft, E. Zudaire, S. Portal-Núñez, F. Cuttitta, and S. B. Jakowlew, "Differentially Expressed Nucleolar Transforming Growth Factor-β1 Target (DENTT) Exhibits an Inhibitory Role on Tumorigenesis," *Carcinogenesis* 29, no. 6 (2008): 1282–1289, https://doi.org/10.1093/carcin/bgn087.

17. X. Zhang, X. Wu, W. Yao, and Y.-H. Wang, "A Tumor-Suppressing Role of TSPYL2 in Thyroid Cancer: Through Interacting With SIRT1 and Repressing SIRT1/AKT Pathway," *Experimental Cell Research* 432, no. 1 (2023): 113777, https://doi.org/10.1016/j.yexcr.2023.113777.

18. H. Tan, M. X. Miao, R. X. Luo, et al., "TSPYL1 as a Critical Regulator of TGFβ Signaling Through Repression of TGFBR1 and TPYL2," *Advanced Science (Weinheim, Baden-Wurttemberg, Germany)* 11, no. 21 (2024): e2306486, https://doi.org/10.1002/advs.202306486.

19. Z. Tang, B. Kang, C. Li, T. Chen, and Z. Zhang, "GEPIA2: An Enhanced Web Server for Large-Scale Expression Profiling and Interactive Analysis," *Nucleic Acids Research* 47, no. W1 (2019): W556–W560, https://doi.org/10.1093/nar/gkz430.

20. T.-Y. Kim, S. Zhong, C. R. Fields, J. H. Kim, and K. D. Robertson, "Epigenomic Profiling Reveals Novel and Frequent Targets of Aberrant DNA Methylation-Mediated Silencing in Malignant Glioma," *Cancer Research* 66, no. 15 (2006): 7490–7501, https://doi.org/10.1158/0008-5472.CAN-05-4552.

21. Y. Li and Y. F. C. Lau, "Tspy and Its X-Encoded Homologue Interact With Cyclin B But Exert Contrasting Functions on Cyclin-Dependent Kinase 1 Activities," *Oncogene* 27, no. 47 (2008): 6141–6150, https://doi.org/10.1038/onc.2008.206.

22. Z. Liu, C. Li, C. Yu, Z. Chen, C. Zhao, and L. Ye, "TSPYL2 Reduced Gefitinib Resistance and DNA Damage Repair via Suppressing SIRT1-Mediated FOXO3 Deacetylation," *Future Medicinal Chemistry* 14, no. 6 (2022): 407–419, https://doi.org/10.4155/fmc-2021-0136.

23. S. Zhang, X. Tong, S. Liu, et al., "AAV9-Tspyl2 Gene Therapy Retards Bleomycin-Induced Pulmonary Fibrosis by Modulating Downstream TGF-β Signaling in Mice," *Cell Death & Disease* 14, no. 6 (2023): 389, https://doi.org/10.1038/s41419-023-05889-8.

24. L. Rahib, B. D. Smith, R. Aizenberg, A. B. Rosenzweig, J. M. Fleshman, and L. M. Matrisian, "Projecting Cancer Incidence and Deaths to 2030: The Unexpected Burden of Thyroid, Liver, and Pancreas Cancers in the United States," *Cancer Research* 74, no. 11 (2014): 2913–2921, https://doi.org/10.1158/0008-5472.CAN-14-0155.

25. A. W. Lambert, D. R. Pattabiraman, and R. A. Weinberg, "Emerging Biological Principles of Metastasis," *Cell* 168, no. 4 (2017): 670–691, https://doi.org/10.1016/j.cell.2016.11.037.

26. J. Massagué and K. Ganesh, "Metastasis-Initiating Cells and Ecosystems," *Cancer Discovery* 11, no. 4 (2021): 971–994, https://doi.org/10.1158/2159-8290.CD-21-0010.

27. P. S. Steeg, "Targeting Metastasis," *Nature Reviews Cancer* 16, no. 4 (2016): 201–218, https://doi.org/10.1038/nrc.2016.25.

28. D. Pan, Q. Zhu, and K. Luo, "Snon Functions as a Tumour Suppressor by Inducing Premature Senescence," *EMBO Journal* 28, no. 22 (2009): 3500–3513, https://doi.org/10.1038/emboj.2009.250.

29. T. Shinagawa, T. Nomura, C. Colmenares, M. Ohira, A. Nakagawara, and S. Ishii, "Increased Susceptibility to Tumorigenesis of Ski-Deficient Heterozygous Mice," *Oncogene* 20, no. 56 (2001): 8100–8108, https://doi.org/10.1038/sj.onc.1204987.

30. J. Deheuninck and K. Luo, "Ski and SnoN, Potent Negative Regulators of TGF-β Signaling," *Cell Research* 19, no. 1 (2009): 47–57, https://doi.org/10.1038/cr.2008.324.

31. K. Walldén, T. Nyman, and B. M. Hällberg, "SnoN Stabilizes the SMAD3/SMAD4 Protein Complex," *Scientific Reports* 7 (2017): 46370, https://doi.org/10.1038/srep46370.

32. Y. Gu, Y. Ji, H. Jiang, and G. Qiu, "Clinical Effect of Driver Mutations of KRAS, CDKN2A/P16, TP53, and SMAD4 in Pancreatic Cancer: A Meta-Analysis," *Genetic Testing and Molecular Biomarkers* 24, no. 12 (2020): 777–788, https://doi.org/10.1089/gtmb.2020.0078.

33. A. C. Tecalco-Cruz, D. G. Ríos-López, G. Vázquez-Victorio, R. E. Rosales-Alvarez, and M. Macías-Silva, "Transcriptional Cofactors Ski and SnoN Are Major Regulators of the TGF-β/Smad Signaling Pathway in Health and Disease," *Signal Transduction and Targeted Therapy* 3 (2018): 15, https://doi.org/10.1038/s41392-018-0015-8.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.