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Expression of ZKSCAN3 protein suppresses proliferation, migration, and invasion of pancreatic cancer through autophagy

Keisuke Nonoyama | Yoichi Matsuo | Saburo Sugita | Yuki Eguchi | Yuki Denda | Hiromichi Murase | Tomokatsu Kato | Hiroyuki Imafuji | Kenta Saito | Mamoru Morimoto | Ryo Ogawa | Hiroki Takahashi | Akira Mitsui | Masahiro Kimura | Shuji Takiguchi

Department of Gastroenterological Surgery, Nagoya City University Graduate School of Medical Sciences, Nagoya, Aichi, Japan

Correspondence
Yoichi Matsuo, Department of Gastroenterological Surgery, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya, Aichi 467-8601, Japan.
Email: matsuo@med.nagoya-cu.ac.jp

Abstract

Elevated autophagy activity enhances the malignancy of pancreatic cancer (PaCa), and autophagy is recognized as a novel therapeutic target. Zinc finger protein with KRAB and SCAN domains 3 (ZKSCAN3) is a transcription factor that suppresses autophagy, but its association with PaCa is unknown. We analyzed the function of ZKSCAN3 in PaCa and investigated whether autophagy regulation through ZKSCAN3 could become a new therapeutic target for PaCa. Using reverse transcription-quantitative polymerase chain reaction and western blotting, we observed that ZKSCAN3 expression was upregulated in several PaCa cell lines compared with normal pancreatic ductal epithelial cells. Additionally, comparing ZKSCAN3 expression with the prognosis of PaCa patients using web databases, we found that higher ZKSCAN3 expression in PaCa was associated with extended overall survival. Knocking down *ZKSCAN3* promoted the proliferation of PaCa cells. Moreover, following *ZKSCAN3* knockdown, PaCa cells exhibited significantly enhanced migratory and invasive properties. Conversely, overexpression of ZKSCAN3 significantly suppressed the proliferation, migration and invasion of PaCa cells. Additionally, the knockdown of *ZKSCAN3* increased the expression of LC3-II, a marker of autophagy, whereas *ZKSCAN3* overexpression decreased LC3-II expression. In a xenograft mouse model, tumors formed by MIA PaCa-2 cells in which *ZKSCAN3* was knocked down significantly increased in size compared with the control group. In conclusion, ZKSCAN3 expression was upregulated in several pancreatic cancer cells. Additionally, it was revealed that *ZKSCAN3* is negatively correlated with the malignancy of PaCa through autophagy. These results suggest that autophagy regulation via *ZKSCAN3* may be a new therapeutic target for PaCa.

Abbreviations: HPDE, human pancreatic ductal epithelial; LC3, microtubule-associated protein 1A/1B-light chain 3; OE-ZKSCAN3, ZKSCAN3 overexpression; OS, overall survival; PaCa, pancreatic cancer; RFS, relapse-free survival; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; shControl, control short hairpin RNA; *shZKSCAN3*, *ZKSCAN3* short hairpin RNA; Vec, empty vector; ZKSCAN3, zinc finger protein with KRAB and SCAN domains 3.

KEYWORDS
autophagy, cancer, pancreatic cancer, zinc finger protein, ZKSCAN3

1 | INTRODUCTION

For patients diagnosed with PaCa, the prognosis is quite poor, with a 5-year survival rate of only ~10%.[1,2] For 2023, it was estimated that there would be 64,050 new cases of PaCa in the United States, resulting in 50,550 pancreatic cancer-related deaths.[2] The mortality rate for PaCa patients is on the rise and, in the United States, pancreatic cancer-related deaths from PaCa are currently rank third but are projected to become the second leading cause of cancer-related deaths by 2030.[3] In Japan, the incidence and mortality rate of PaCa have been steadily increasing since the 1960s,[4] underscoring the urgent need for the development of novel approaches to the treatment of PaCa.

The clinical aggressiveness of PaCa is attributed to its early metastasis to adjacent tissues and distant organs, as well as resistance to chemotherapy and radiation therapy.[5–7] Recently, it was recognized that macroautophagy (hereafter referred to as autophagy) plays a significant role in carcinogenesis, cancer progression and the development of drug resistance.[8,9] Autophagy is essential for maintaining homeostasis within the body. It serves as a major cellular protective mechanism by removing damaged components and recycling nutrients, thereby contributing to energy homeostasis under both normal and stressful conditions.[10] In general, autophagy acts as a suppressive mechanism for carcinogenesis in normal cells. However, once cells become cancerous, autophagy supports the metabolism of cancer cells, promoting their survival and progression.[8,11] In PaCa cells, reports have indicated that sustained activation of autophagy is associated with the progression of PaCa, and it is considered a potential new therapeutic target for PaCa.[8,12,13]

ZKSCAN3 belongs to the zinc finger transcription factor family with a Kruppel-associated box (KRAB) and SCAN domains.[10] The KRAB-containing protein family binds to DNA through C2H2 zinc finger domains, and the KRAB domain functions as a strong transcriptional repression domain.[14] The biochemical functions of KRAB-containing proteins are believed to be important in various intracellular roles, including cell differentiation, cell proliferation, apoptosis, and neoplastic transformation.[14] Additionally, ZKSCAN3 acts as a major inhibitory transcription factor for autophagy.[10,15] ZKSCAN3 regulates the expression of more than 60 genes that encode proteins involved in various stages of autophagy and lysosome biosynthesis/function, such as *Map1lc3b*, *Ulk1*, and *Wipi2*.[10,15] Moreover, the inhibition of autophagy by overexpression of *ZKSCAN3* reportedly suppresses tumor progression.[16] Conversely, ZKSCAN3 protein also acts as a tumor-promoting factor and enhances cancer cell progression and migration in various tumors,[17] including colorectal cancer,[18,19] breast cancer,[20] bladder cancer,[21] prostate cancer,[22] prostate cancer,[23] gastric cancer,[24] and cervical cancer.[25] In these reports, the growth impairment associated with silencing *ZKSCAN3* is also believed to partially reflect an increase in autophagy leading to cellular senescence.[10] While previous research has reported associations between ZKSCAN3 and various cancer types, there have been no reports studying the function of ZKSCAN3 in PaCa.

In this study, we investigated how *ZKSCAN3* gene expression functions in PaCa and explored whether the ZKSCAN3 protein is associated with autophagy in PaCa. Targeting key regulators of autophagy in PaCa could represent a promising new strategy for the treatment of PaCa.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Six human PaCa cell lines (AsPC-1, BxPC-3, Capan-2, MIA PaCa-2, PANC-1, and SW1990) were purchased from the ATCC. The HPDE cell line, H6c7, was purchased from Kerafast, Inc. AsPC-1, BxPC-3 and Capan-2 cell lines were maintained in RPMI-1640 medium (Sigma Aldrich; Merck KGaA). MIA PaCa-2, PANC-1 and SW1990 cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Aldrich; Merck KGaA). The H6c7 cell line was maintained in a keratinocyte serum-free medium (Gibco/Thermo Fisher Scientific, Inc.). Fetal bovine serum (FBS; Gibco/Thermo Fisher Scientific, Inc.) was added to both RPMI-1640 medium and DMEM to achieve a concentration of 10%. All media were supplemented with 10 mg/mL streptomycin, 10,000 U/mL penicillin, and 25 μg/mL amphotericin B (Gibco/Thermo Fisher Scientific, Inc.). All cell lines were cultured at 37°C in a humidified incubator containing 5% CO_2.

2.2 | Stable cell lines

We transfected a human *ZKSCAN3*-targeting short hairpin RNA (shRNA) plasmid (sc-95,093; Santa Cruz Biotechnology, Inc.) or a control-shRNA plasmid (sc-108,060; Santa Cruz Biotechnology, Inc.) into pancreatic cancer cell lines PANC-1, MIA PaCa-2, and SW 1990 using Plasmid Transfection Reagent (Santa Cruz Biotechnology, Inc.). Selection of stable clones was carried out by treating each PaCa cell line with puromycin (CAS 58–58-2; cat. no. sc-108,071; Santa Cruz Biotechnology, Inc.) at the concentrations stated below: Specifically, PANC-1 was treated at 5 μg/mL, MIA PaCa-2 at 1 μg/mL, and SW 1990 at 1 μg/mL. Conversely, the *ZKSCAN3* expression construct (RC202971; OriGene Technologies, Inc.) or empty vector (PS100001; OriGene Technologies, Inc.) was

transfected into the pancreatic cancer cell line BxPC-3 using jetOPTIMUS (Funakoshi Co., Ltd.). To obtain stable clones, these cells were treated with 100 μg/mL G418 (cat. no. 10131035; Gibco/Thermo Fisher Scientific, Inc.).

2.3 | Cell proliferation assay

To assess cell growth, we used the Premix WST-1 Cell Proliferation Assay System (Takara Bio, Inc.) according to the manufacturer's protocol. PANC-1, MIA PaCa-2, and SW 1990 pancreatic cancer cell lines that expressed high levels of *ZKSCAN3* were gene-silenced by transfection of *ZKSCAN3*-shRNA or control-shRNA. In addition, we used the pancreatic cancer cell line BxPC-3, which expresses low levels of *ZKSCAN3*. It was treated by transfection with a *ZKSCAN3* construct or empty vector. In brief, PaCa cells (1×10^4) were seeded into 12-well plates and incubated for 1–5 days at 37°C. The absorbance was measured at 1, 3, and 5 days after cell seeding. After cultivation, 100 μL of WST-1 solution was added to each well with 1000 μL of medium for 1 h at 37°C. We transferred 100 μL of each solution into separate wells of 96-well plates and measured the absorbance at a wavelength of 450 nm with a background subtraction at 650 nm in each well using the SpectraMax 340 spectrophotometer from Molecular Devices, LLC.

2.4 | Colony formation assay

PANC-1, SW 1990, and BxPC-3 cells were seeded at 1000 cells per 60-mm dish, whereas MIA PaCa-2 cells were seeded at 500 cells each. The cells were then cultured at 37°C for 14 days. After fixation, cell staining was performed using the Diff-Quick cell staining kit (Dade Behring). Five different fields of colonies were counted per dish. A colony was defined as a group of at least 50 cells.

2.5 | Reverse transcription-quantitative polymerase chain reaction

Total RNA was extracted from HPDH and PaCa cells using QIAcube and the RNeasy Plus Mini Kit (Qiagen GmbH), following the manufacturer's protocol. Total RNA was subsequently quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.). The RNA was then reverse transcribed using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen; Thermo Fisher Scientific, Inc.) and the T100 Thermal Cycler (Bio-Rad Laboratories, Inc.), following the manufacturer's protocol. The temperature protocol for reverse transcription was as follows: 25°C for 10 min, 50°C for 30 min and 85°C for 5 min. RT-qPCR was performed using TaqMan Fast Advanced Master Mix and TaqMan Gene Expression Assays for *ZKSCAN3* (Hs01069653_s1) and *GAPDH* (Hs99999905_m1), on a CFX Connect Real-Time System (Bio-Rad Laboratories, Inc.). The thermocycling conditions were as follows: initial denaturation at 95°C for 20 s, followed by 60 cycles at 95°C for 1 s and 60°C for 20 s. The expression level of *ZKSCAN3* was reported relative to that of *GAPDH* in each sample, using the relative standard curve method.[26]

2.6 | Western blotting

We used RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Inc.; cat. no. 89900) containing Protease Inhibitor Single Use Cocktail and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Inc) to extract proteins from cells. The protein concentration was measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Inc.). The protein extracts (30 μg) were denatured at 90°C for 5 min and separated on a 10% Mini-PROTEAN TGX Precast Gel (Bio-Rad Laboratories). The protein bands were then transferred onto a nitrocellulose membrane. Blocking and incubation with primary and secondary antibodies were performed using the iBind Flex Western System (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions at room temperature for 4 h. The membrane was incubated with a primary antibody against ZKSCAN3 (1:500; Santa Cruz Biotechnology; sc-515,285), followed by incubation with horseradish peroxidase-conjugated goat anti-mouse polyclonal secondary antibodies (1:2000; DAKO/Agilent Technologies; cat. no. P0447). For anti-GAPDH primary antibodies (1:1000; Cell Signaling Technology; #2118), horseradish peroxidase-conjugated goat anti-rabbit polyclonal secondary antibodies (1:2000; DAKO/Agilent Technologies; cat. no. P0448) were used. Protein–antibody complexes were visualized using SuperSignal West Femto Chemiluminescent Substrate, SuperSignal West Pico PLUS Chemiluminescent Substrate or Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.). Immunoreactive protein bands were detected using the Amersham Imager 600 (Cytiva), and the densities of the detected bands were calculated using ImageJ software 1.52v (National Institutes of Health).

2.7 | Wound healing assay

PaCa cells ($0.5–2\times10^5$) were seeded in 24-well plates and cultured at 37°C for 24 h. When the cells reached at least 90% confluency, a straight scratch was made on the monolayer of cells using a sterile P200 pipette tip, and the medium was replaced with a medium containing 2% FBS. Immediately after the scratch, photographs were taken. Subsequently, the cells were cultured at 37°C, and for PANC-1, images were taken 18 h after scratching, whereas for MIA PaCa-2, SW 1990, and BxPC-3, images were taken 24 h after scratching, all at the same positions as the initial scratch. Imaging was performed using a BZ-X710 fluorescence microscope (Keyence Corporation, Osaka, Japan). The wound areas were calculated

using ImageJ software 1.52v (National Institutes of Health). The percentage of the wound area that filled was calculated using the following formula:

$$\%\text{ of wound area filled} = (S_0 - S_a) / S_0 \times 100,$$

where S_0 and S_a represent the initial wound area and the wound area after culturing, respectively.

2.8 | Transwell migration assay and invasion assay

Transwell migration and invasion assays were performed using the Boyden double chamber method using 24-well plates. In migration assays, we used Falcon Cell Culture Inserts (pore size, 8.0 μm diameter). In invasion assays, we used Corning BioCoat Matrigel Invasion Chambers (Corning, Inc.) according to the manufacturer's protocol. PaCa cells (1×10^5) were seeded in the upper chamber, which contained 500 μL serum-free medium. In the lower chamber, 750 μL of medium supplemented with 10% FBS was added to act as a chemoattractant. After 24 h of incubation at 37°C, the upper surfaces of the upper chambers were gently wiped with a cotton swab, and the invading cells were subsequently fixed and stained using a Diff-Quick cell staining kit (Dade Behring). The number of cells in nine randomly selected microscopic fields (at 100x magnification) was then counted. Percentage invasion was calculated by dividing the number of invading cells passing through the Matrigel-coated inserts by the number of invading cells passing through non-Matrigel-coated inserts.[27]

2.9 | Animals

In total, 10 female 4-week-old BALB/c nu-nu mice were purchased from Japan SLC, Inc. A 2-week adaptation period was provided to acclimate the mice to their environment. The mice were given regular sterilized feed and had unrestricted access to drinking water. They were housed in standard Plexiglas cages (four mice per cage) and maintained in a room with consistent temperature and humidity, as well as a 12-h light–dark cycle. All animal experiments were conducted with the approval of the Animal Welfare and Use Committee of the Graduate School of Medicine, Nagoya City University (approval no. 23-019; Nagoya, Japan). The animal facilities and laboratories in the Center for Laboratory Animal Research and Education, Nagoya City University are equipped with containment measures at the P2A level and certified (certification no. FM3).

2.10 | Experimental protocol

The mice were divided randomly into two groups, each consisting of five mice: the control group and the shZKSCAN3 group. Prior to subcutaneous transplantation into the mice, the sterility of the cells to be transplanted was confirmed using a *Mycoplasma* detection kit provided by Lonza Group, Ltd.

As a general anesthetic, 2% isoflurane (product no. 099–06571; FujiFilm Wako Pure Chemical Corporation) was administered using a vaporizer (Rodent Circuit Controller; Vetequip, Inc.). MIA PaCa-2 cells (5×10^6 in 200 μL of Hanks's Balanced Salt Solution, purchased from Sigma Aldrich, product code H9269) were injected subcutaneously into the right flank of each mouse. Tumor volume was measured once a week using calipers (Shinwa Co., Ltd.). Tumor volume was calculated using the following formula:

Tumor volume (mm^3) = $(A \times B^2)/2$, where A represents the longest diameter of the tumor, and B represents the shortest diameter, both measured in millimeters. Mice were sacrificed 4 weeks after subcutaneous injection of the tumor. Finally, the tumors were removed from the mice, fixed in formaldehyde and stored for further analysis.

2.11 | Immunohistochemical examination

Subcutaneous xenograft tumors were fixed with paraformaldehyde and embedded in paraffin. The sections were mounted on slides coated with 3-aminopropyltriethoxysilane. Immunohistochemical staining was performed using the Leica Biosystems BOND RXm, following the manufacturer's protocol, in an automated manner. The Compact Polymer detection system used BOND Polymer Refine Detection (DS9800), which contains a peroxide block, post primary, polymer reagent, DAB chromogen and hematoxylin counterstain. The primary antibodies were as follows: a rabbit polyclonal anti-ZKSCAN3 antibody (ab187866; 1:100; Abcam) and a rabbit polyclonal anti-LC3 antibody (PM036; 1:2000; Medical & Biological Laboratories Co., Ltd), a rabbit monoclonal anti-Ki-67 antibody (SP6; code 418071; 1:2; Nichirei Biosciences, Inc.). Images of the stained slides were captured using the BZ-X710 fluorescence microscope (Keyence Corporation, Osaka, Japan). The stained areas of ZKSCAN3 and LC3 were quantified using the hybrid cell count software (BZ-H4C; Keyence Corporation, Osaka, Japan). The Ki-67 labeling index was generated by counting 1000 tumor cells in the hot spot and expressed as a percentage of positive tumor cells.

2.12 | Statistical analysis

Statistical analysis was performed using EZR software (Easy R) version 1.41 (https://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmed.html; Saitama Medical Center, Jichi Medical University, Saitama, Japan). In vitro experiments were typically conducted at least three times, while in vivo experiments were performed only once. All data are presented as the mean ± standard deviation. Two groups were compared using unpaired Student's *t*-test, whereas two or more groups were compared using one-way ANOVA followed by the Bonferroni test. A p-value < 0.05 was defined as indicating a statistically significant difference.

3 | RESULTS

3.1 | ZKSCAN3 was expressed at higher levels in PaCa cells than in human pancreatic ductal epithelial cells

H6c7 is an immortalized epithelial cell line derived from normal human pancreatic duct epithelial (HPDE) cells. The expression levels of *ZKSCAN3* mRNA and ZKSCAN3 protein in H6c7 cells and PaCa cell lines (AsPC-1, BxPC-3, Capan-2, MIA PaCa-2, PANC-1 and SW1990) were evaluated by RT-qPCR and western blotting. *ZKSCAN3* mRNA expression (Figure 1a) was significantly higher in multiple PaCa cell lines (PANC-1, SW1990, AsPC-1) than in HPDE cells. At the protein expression level (Figure 1b), ZKSCAN3 was also significantly elevated in several PaCa cells (PANC-1, SW1990, AsPC-1 and MIA PaCa-2). Additionally, we confirmed the expression levels of *ZKSCAN3* in PaCa tissues and normal pancreatic tissues using an online database (https://xena.ucsc.edu, accessed February 20, 2024). Gene expression data from the UCSC Xena website were sourced from The Cancer Genome Atlas, Therapeutically Applicable Research to Generate Effective Treatments, and Genotype Tissue Expression. As shown here, *ZKSCAN3* mRNA expression was significantly higher in PaCa tissues compared with normal pancreatic tissues ($p < 0.01$) (Figure 1c).

3.2 | In PaCa, the high ZKSCAN3 expression group has a better prognosis compared with the low ZKSCAN3 expression group

We confirmed the clinical significance of *ZKSCAN3* expression in PaCa using an online database (https://kmplot.com/analysis/index.php?p=service&cancer=pancancer_rnaseq, accessed May 28, 2022). Gene expression data from the Kaplan–Meier plotter website were sourced from NCBI Gene Expression Omnibus, The European

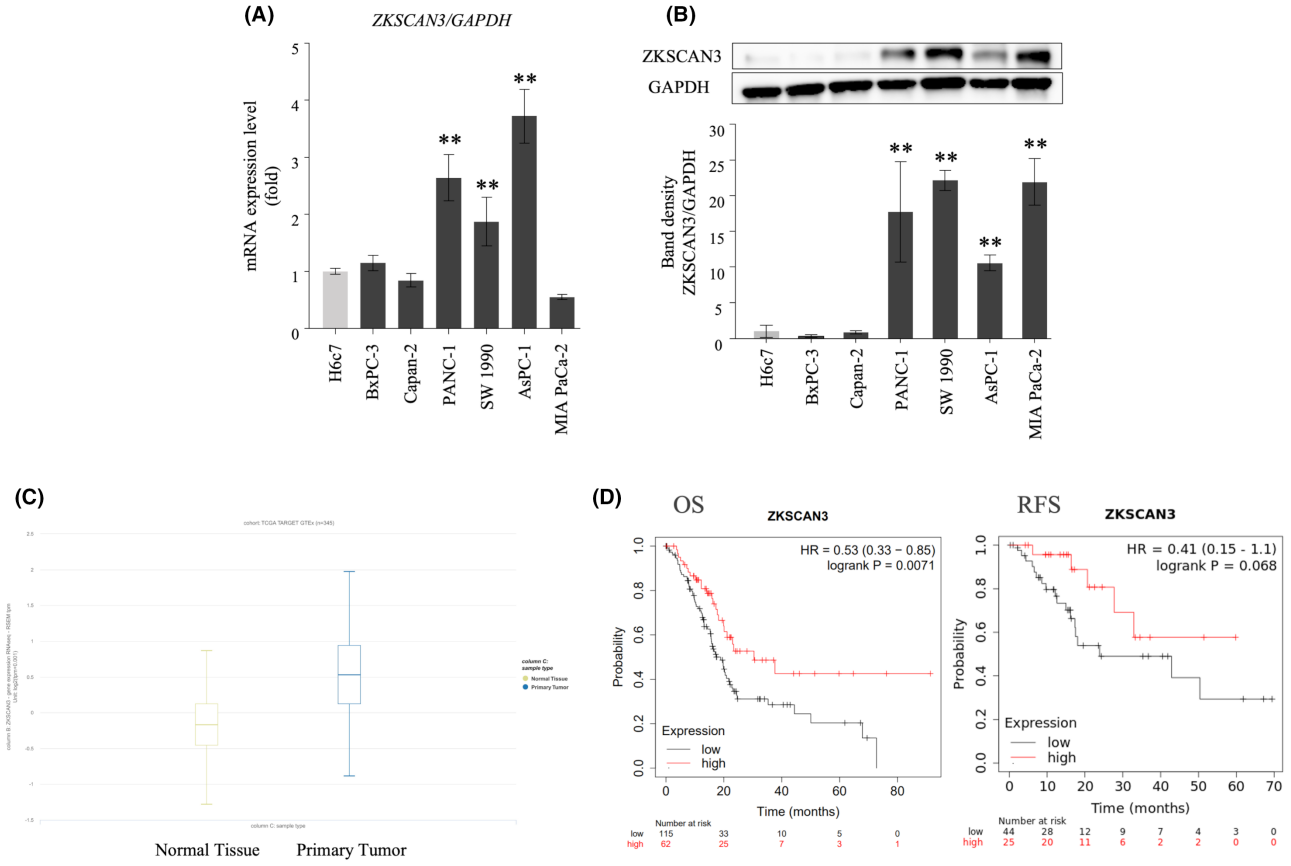

FIGURE 1 *ZKSCAN3* expression in PaCa cells and its relationship to the prognosis of PaCa patients. (A) *ZKSCAN3* mRNA expression in HPDE cell line (H6c7) and PaCa cell lines (BxPC-3, Capan-2, PANC-1, SW1990, AsPC-1, and MIA PaCa-2) were assessed using RT-qPCR. The mRNA expression levels in each sample were normalized to the expression of *GAPDH*. (B) ZKSCAN3 protein expression levels in the HPDE cell line (H6c7) and PaCa cell lines (BxPC-3, Capan-2, PANC-1, SW1990, AsPC-1, and MIA PaCa-2) were evaluated using western blotting. The band densities of ZKSCAN3 in western blots from each cell line were calculated relative to the band densities of GAPDH. Comparisons between HPDE cells and PaCa cell lines were assessed using one-way analysis of variance. (C) The box plots illustrate the mRNA expression levels of *ZKSCAN3* in PaCa tissues and normal pancreatic tissues obtained from the database in the UCSC Xena website (https://xena.ucsc.edu, accessed 20 February 2024). (D) Kaplan–Meier curves depict OS of 177 PaCa patients and RFS of 69 PaCa patients from the database in the Kaplan–Meier plot website (https://kmplot.com/analysis/index.php?p=service&cancer=pancancer_rnaseq, accessed May 28, 2022). $^{*}p < 0.05$; $^{**}p < 0.01$ compared with H6c7.

Genome-phenome Archive, and The Cancer Genome Atlas.[28] *ZKSCAN3* gene expression was evaluated at the mRNA level. As shown here, strong expression of *ZKSCAN3* in PaCa was significantly associated with favorable OS, and while there is no significant difference in RFS, there is a trend toward better prognosis (Figure 1d).

3.3 | ZKSCAN3 mRNA and ZKSCAN3 protein expression levels after ZKSCAN3 knockdown in PaCa cell lines

For the transfection of *ZKSCAN3* shRNA, we selected PANC-1, MIA PaCa-2, and SW 1990 cells, which exhibit high protein-level expression of ZKSCAN3. To assess the changes in *ZKSCAN3* gene expression and ZKSCAN3 protein expression in PaCa cell lines transfected with *ZKSCAN3* shRNA, we performed RT-qPCR and western blotting. PaCa cell lines transfected with *shZKSCAN3* demonstrated significant downregulation of *ZKSCAN3* compared with cells transfected with shControl (Figure 2a–c).

3.4 | Knockdown of ZKSCAN3 enhanced the proliferation of PaCa cells

Cell proliferation assays and colony formation assays were performed to evaluate the proliferative potential of PaCa cell lines.

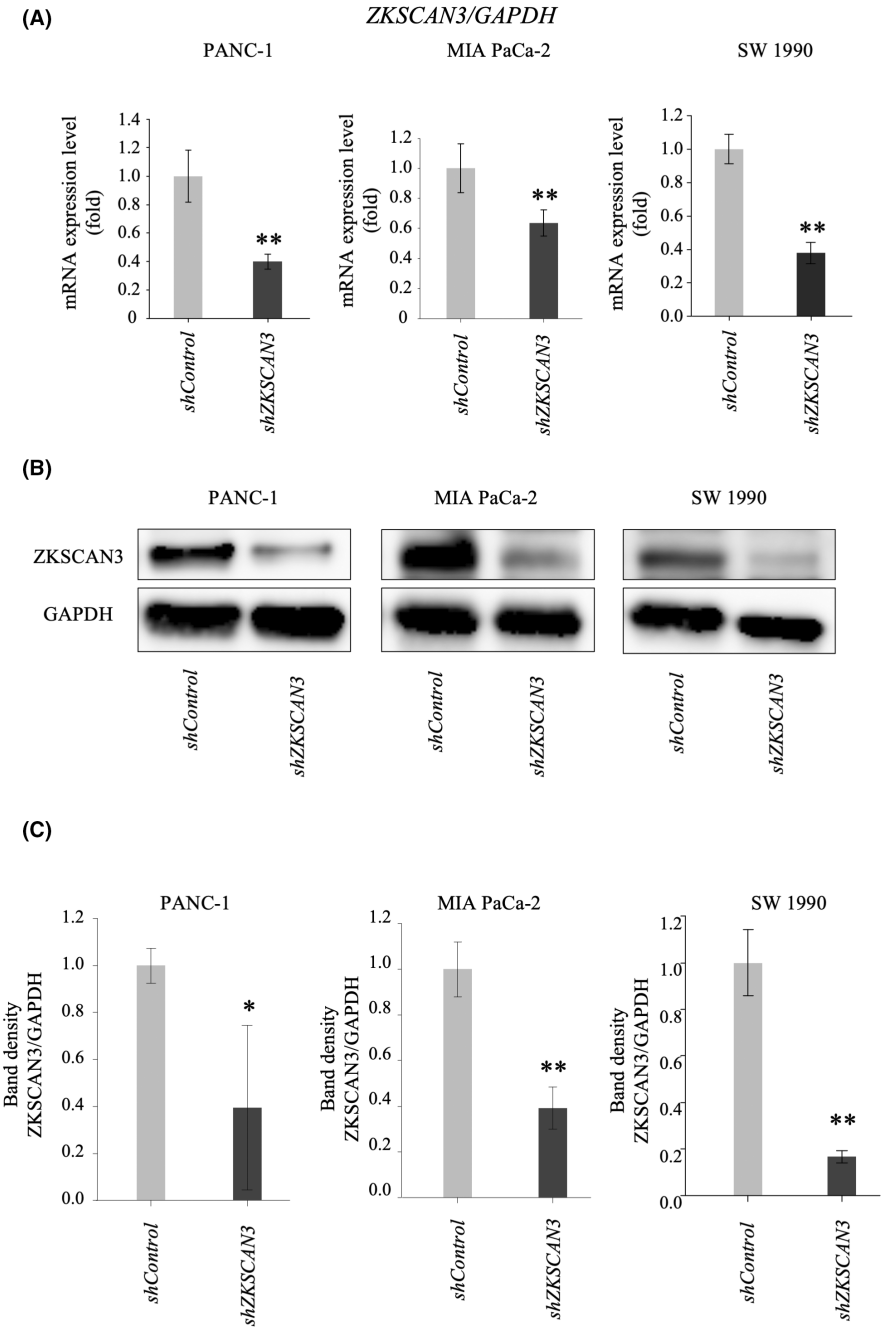

FIGURE 2 Knockdown of *ZKSCAN3* in PaCa cell lines with high *ZKSCAN3* expression. (A) *ZKSCAN3* mRNA expression after transfection with *ZKSCAN3* shRNA was assessed using RT-qPCR. The mRNA expression levels in each sample were normalized to the expression of *GAPDH*. (B) ZKSCAN3 protein expression after transfection with *ZKSCAN3* shRNA was evaluated using western blotting. (C) The band densities of ZKSCAN3 in western blotting in each PaCa cell line were calculated relative to the band density of GAPDH. Comparisons between shControl cells and *shZKSCAN3* cells were assessed using *t*-tests. $^{*}p<0.05$; $^{**}p<0.01$ compared with each shControl group.

The proliferative ability of PaCa cells was significantly enhanced in the *ZKSCAN3* knockdown group compared with the shControl group (Figure 3a). Furthermore, the *ZKSCAN3* knockdown group showed significantly increased colony formation by PaCa cells compared with the shControl group (Figure 3b,c).

3.5 | Knockdown of ZKSCAN3 protein enhanced the migration of PaCa cells

Wound healing assay and Transwell migration assay were performed to evaluate the migratory ability of PaCa cell lines. The *ZKSCAN3*-knockdown PaCa group showed significantly enhanced migratory ability compared with the shControl group (Figure 4a,b). Furthermore, in the Transwell migration assay, the migratory ability of PaCa cells was significantly enhanced in the *ZKSCAN3*- knockdown group compared with the shControl group (Figure 5a,b).

3.6 | Knockdown of ZKSCAN3 enhanced invasion of PaCa cells

Matrigel invasion assays were performed to evaluate the invasive ability of PaCa cell lines. The number of PaCa cells that migrated through the Matrigel-coated inserts was significantly increased in the *ZKSCAN3*-knockdown group compared with the shControl group (Figure 5a,b). We calculated the Percentage Invasion to assess changes in cell invasive capability. We observed that the knockdown of *ZKSCAN3* significantly enhanced the invasive ability of PaCa cells in both PANC-1 and MIA PaCa-2 cell lines (Figure 5b). However, in SW 1990 cells, there was no change in the invasive capacity of the *shZKSCAN3* group relative to the shControl group (Figure 5b). Using western blotting, we assessed the alterations in matrix metalloproteinase protein expression following *ZKSCAN3* knockdown. In MIA PaCa-2 cells, *ZKSCAN3* knockdown led to increased expression of both MMP-2 and MMP-9 (Figure 5c–e). In PANC-1 cells, MMP-2 expression was

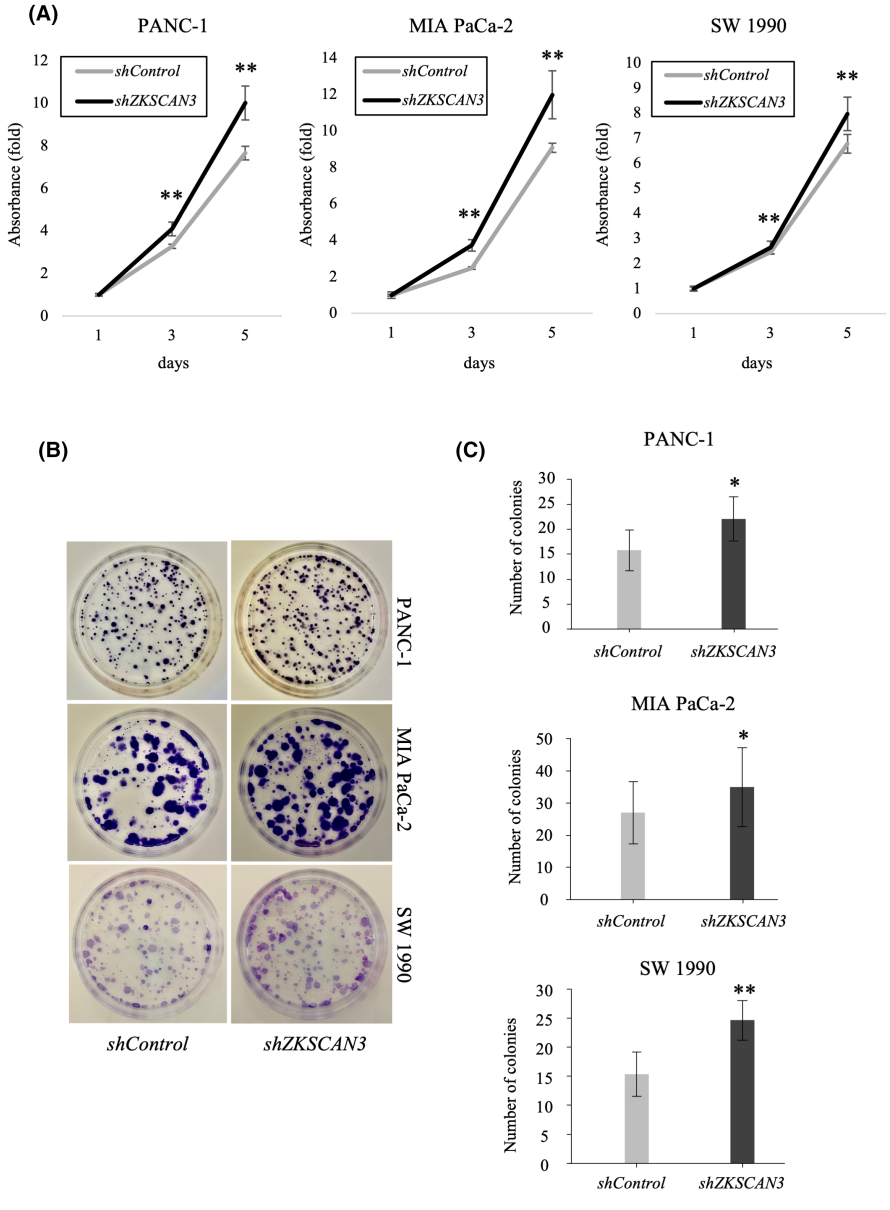

FIGURE 3 Altered proliferative abilities of PaCa cell lines (PANC-1, MIA PaCa-2, SW 1990) after *ZKSCAN3* knockdown. (A) PaCa cells (PANC-1, MIA PaCa-2, SW 1990) were seeded at a density of 10,000 cells per well in 12-well plates and incubated at 37°C. The proliferation abilities of PaCa cells were evaluated using the Premix WST-1 Cell Proliferation Assay System. Absorbance was measured at 1, 3, and 5 days after cell seeding. To assess the growth of adherent cells, each absorbance value was normalized to the absorbance determined 1 day after seeding. (B) Colony formation assay. PANC-1 and SW 1990 cells were seeded at a density of 1000 cells per 60-mm dish, whereas MIA PaCa-2 cells were seeded with 500 cells. Cultures were maintained at 37°C for 14 days. After fixation, the cells were stained. (C) The number of colonies. Five different locations within each dish were counted to quantify the number of colonies formed. $^{*}p < 0.05$; $^{**}p < 0.01$.

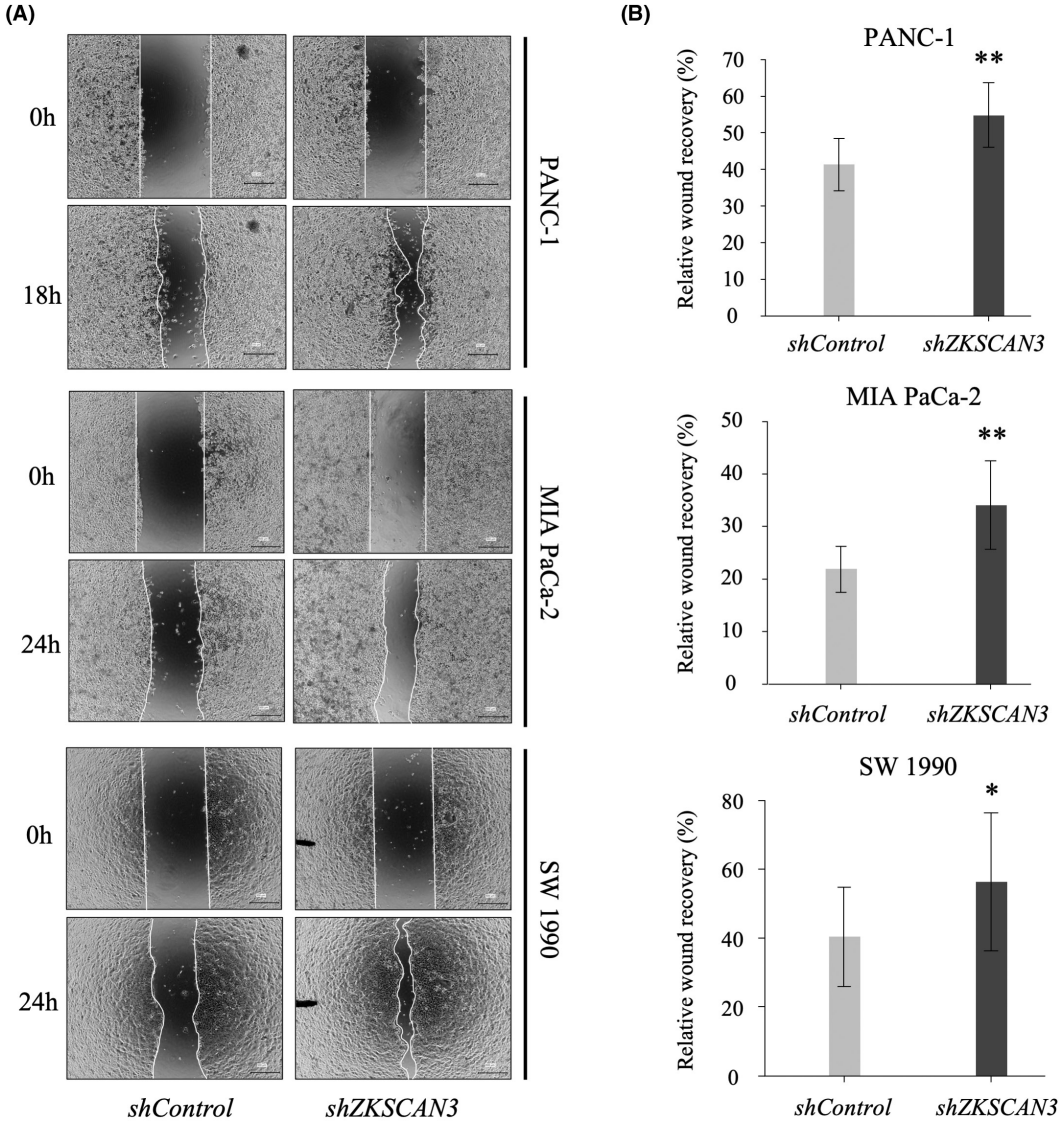

FIGURE 4 Changes in the migration ability of PaCa cell lines after *ZKSCAN3* knockdown. (A) The migratory ability of PaCa cells was assessed using a wound healing assay. Cells ($0.5–2\times10^5$) were seeded in 24-well plates and cultured at 37°C for 24 h. When the cells reached a confluence over 90%, a sterile P200 pipette tip was used to scratch a straight path across the monolayer of cells. The cells were cultured for 18–24 h in medium containing 2% FBS and were subsequently observed under a phase-contrast microscope. Scale bar, 500 μm. (B) The area of the wound was calculated using ImageJ software 1.52v, and the % wound area filled was determined using the following formula: %wound area filled = $(S_0 - S_a)/S_0 \times 100$. Here, S0 represents the initial wound area, and Sa represents the wound area after culture. *, $p<0.05$; **$p<0.01$.

significantly upregulated in the *shZKSCAN3* group, whereas MMP-9 expression remained unchanged (Figure 5c–e). Conversely, in SW 1990 cells, *ZKSCAN3* knockdown did not alter MMP-9 expression but led to a decrease in MMP-2 expression (Figure 5c–e).

3.7 | Overexpression of ZKSCAN3 led to decreased proliferation of PaCa cells

Subsequently, we evaluated changes in the function of PaCa cells when *ZKSCAN3* was overexpressed. Thus, we selected the ZKSCAN3-low-expressing PaCa cell line BxPC-3 and created cell lines overexpressing ZKSCAN3. To assess the changes in these PaCa cell lines overexpressing *ZKSCAN3*, we performed RT-qPCR and western blotting. The PaCa cell lines OE-ZKSCAN3 exhibited a significant upregulation of *ZKSCAN3* compared with cells transfected with a Vec (Figure 6a–c). Additionally, in the OE-ZKSCAN3 group, the proliferative capacity of pancreatic cancer cells was significantly suppressed compared with the Vec group (Figure 6d–f).

3.8 | Overexpression of ZKSCAN3 decreased migration and invasion of PaCa cells

In both the wound healing assay and Transwell migration assay, overexpression of *ZKSCAN3* significantly reduced the migratory

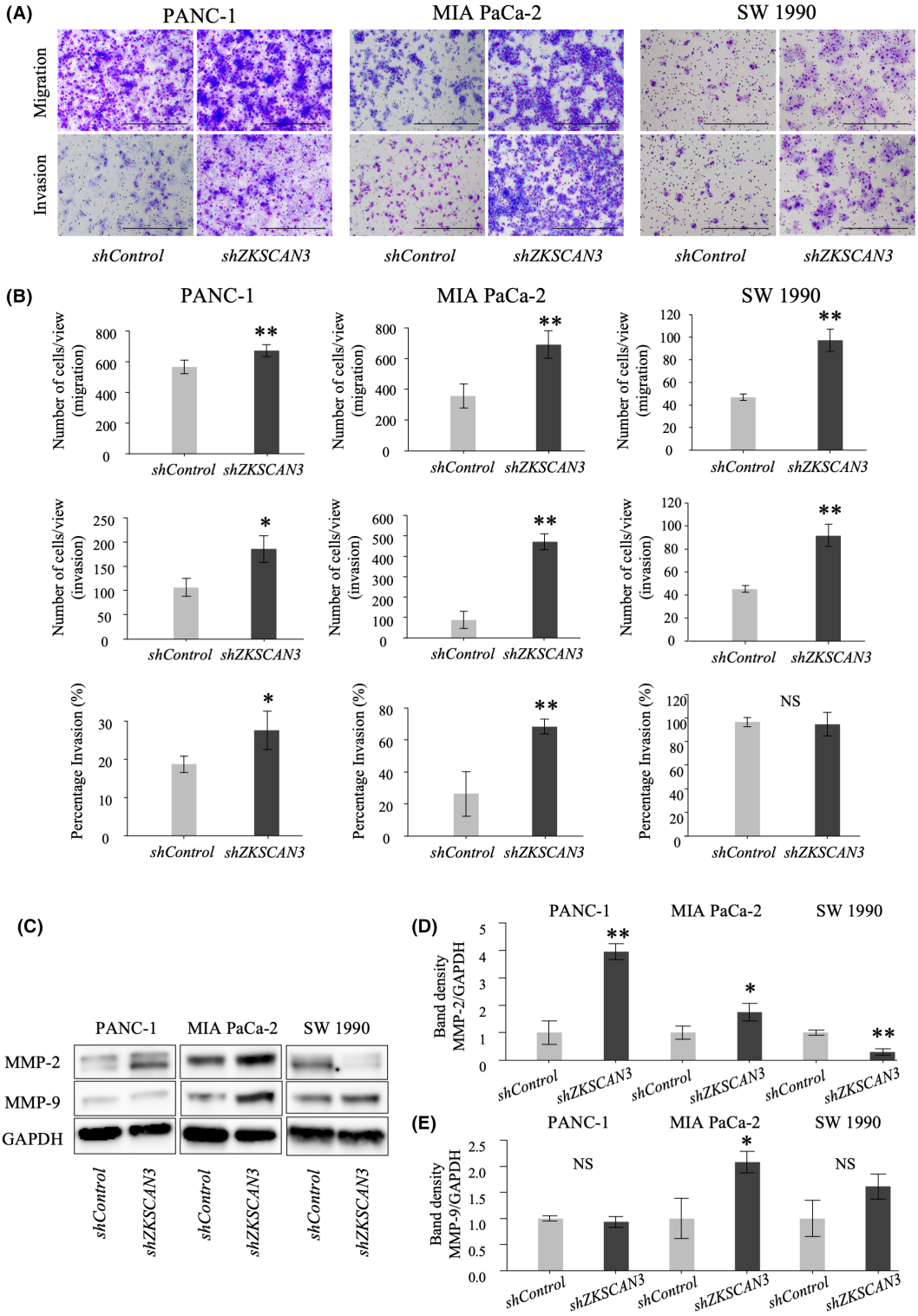

FIGURE 5 Changes in the migratory and invasive abilities and matrix metalloproteinase expression levels in PaCa cell lines after *ZKSCAN3* knockdown. The migratory and invasive abilities of PaCa cells were assessed using the Boyden double chamber method. PaCa cells (1×10^5) were seeded into Transwell chambers and cultured at 37°C for 24 h. For the evaluation of migratory ability, the upper chamber was uncoated, whereas the upper chamber was coated with Matrigel for the evaluation of invasive ability. (A) Cells that invaded through the membrane to the bottom of the upper chamber were observed after fixation and staining. Magnification ×100. Scale bar, 500 μm. (B) The number of invading cells in nine random microscopic fields was quantified. Percentage invasion was calculated by dividing the number of invading cells through Matrigel-coated inserts by the number of invading cells through non-coated inserts. (C) Changes in the expression of MMP-2 and MMP-9 due to *ZKSCAN3* knockdown were evaluated using western blotting. (D, E) Band densities of MMP-2 and MMP-9 in western blots from each cell line were calculated relative to the band density of GAPDH. Comparisons between groups were assessed using unpaired Student's *t*-test. $^*p<0.05$; $^{**}p<0.01$; NS indicates no significant difference compared with the shControl group.

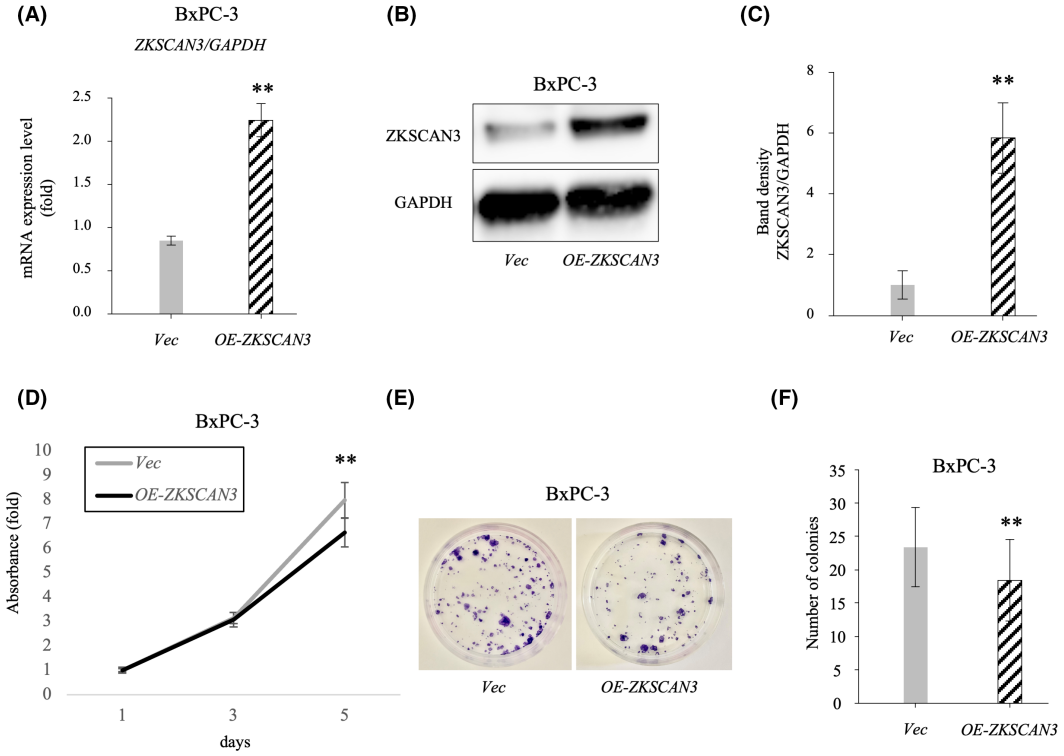

FIGURE 6 Overexpression of *ZKSCAN3* in BxPC-3 cells with low *ZKSCAN3* expression. (A) *ZKSCAN3* mRNA expression after transfection with the *ZKSCAN3* expression construct was assessed using RT-qPCR. The mRNA expression levels for each sample were normalized to *GAPDH* expression. (B) ZKSCAN3 protein expression after transfection with the *ZKSCAN3* expression construct was evaluated using western blotting. (C) Band densities of ZKSCAN3 on western blots from each cell line were calculated relative to the band density of GAPDH. (D) The proliferative ability of BxPC-3 cells was evaluated using the Premix WST-1 Cell Proliferation Assay System. BxPC-3 cells (1×10^4 cells) were seeded in a 12-well plate and cultured at 37°C. Absorbance measurements were taken 1, 3, and 5 days after seeding the cells. To assess the proliferation of adherent cells, each absorbance value was normalized to the absorbance 1 day after seeding the cells. (E) Colony formation assay. BxPC-3 cells were seeded at a density of 1000 cells per 60-mm dish. They were cultured at 37°C for 14 days. After fixation, the cells were stained. (F) The number of colonies per dish. Five different locations within each dish were counted to quantify the number of colonies formed. Comparisons between groups were assessed using unpaired Student's *t*-test. $^{**}p<0.01$.

ability of PaCa cells in both experiments (Figure 7a–c). Furthermore, in the Matrigel invasion assay, *ZKSCAN3* overexpression resulted in a significant decrease in the number of PaCa cells penetrating the Matrigel-coated inserts compared with the Vec group, leading to a significant reduction in Percentage Invasion (Figure 7c,d). Additionally, *ZKSCAN3* overexpression led to a decrease in the expression of both MMP-2 and MMP-9 (Figure 7e–g).

3.9 | ZKSCAN3 expression controlled the expression of the autophagy marker LC3-II

Next, we evaluated the expression of LC3-II using western blotting analysis of PaCa cell lines in which *ZKSCAN3* was either knocked down or overexpressed. In three PaCa cell lines (PANC-1, MIA PaCa-2, SW 1990), knocking down *ZKSCAN3* significantly upregulated the expression of LC3-II compared with the shControl (Figure 8a,b). Conversely, in BxPC-3 cells overexpressing *ZKSCAN3*, the expression of LC3-II was significantly decreased compared with the Vec group (Figure 8c,d).

3.10 | Subcutaneous implantation of ZKSCAN3-knockdown MIA PaCa-2 in a xenograft mouse model enhanced cancer cell proliferation

To assess the effect of the *ZKSCAN3* gene on the in vivo proliferative capacity of PaCa cells, we established a subcutaneous xenograft model of MIA PaCa-2 in nude mice. Tumors were subcutaneously transplanted into both shControl and *shZKSCAN3* groups of mice. Tumor formation was confirmed, and the mice were sacrificed four weeks after subcutaneous transplantation. As shown in Figure 9, the *shZKSCAN3* group exhibited a significant increase in both tumor volume and tumor weight compared with the shControl group (Figure 9a–c). Additionally, immunohistochemical staining of tumor tissues revealed that *ZKSCAN3* expression was suppressed in the *shZKSCAN3* group compared with the shControl group (Figure 9d,e). Furthermore, the expression of LC3-II was significantly upregulated in the *shZKSCAN3* group (Figure 9d,f). The Ki-67 labeling index was significantly elevated in the *shZKSCAN3* group (Figure 9d,g).

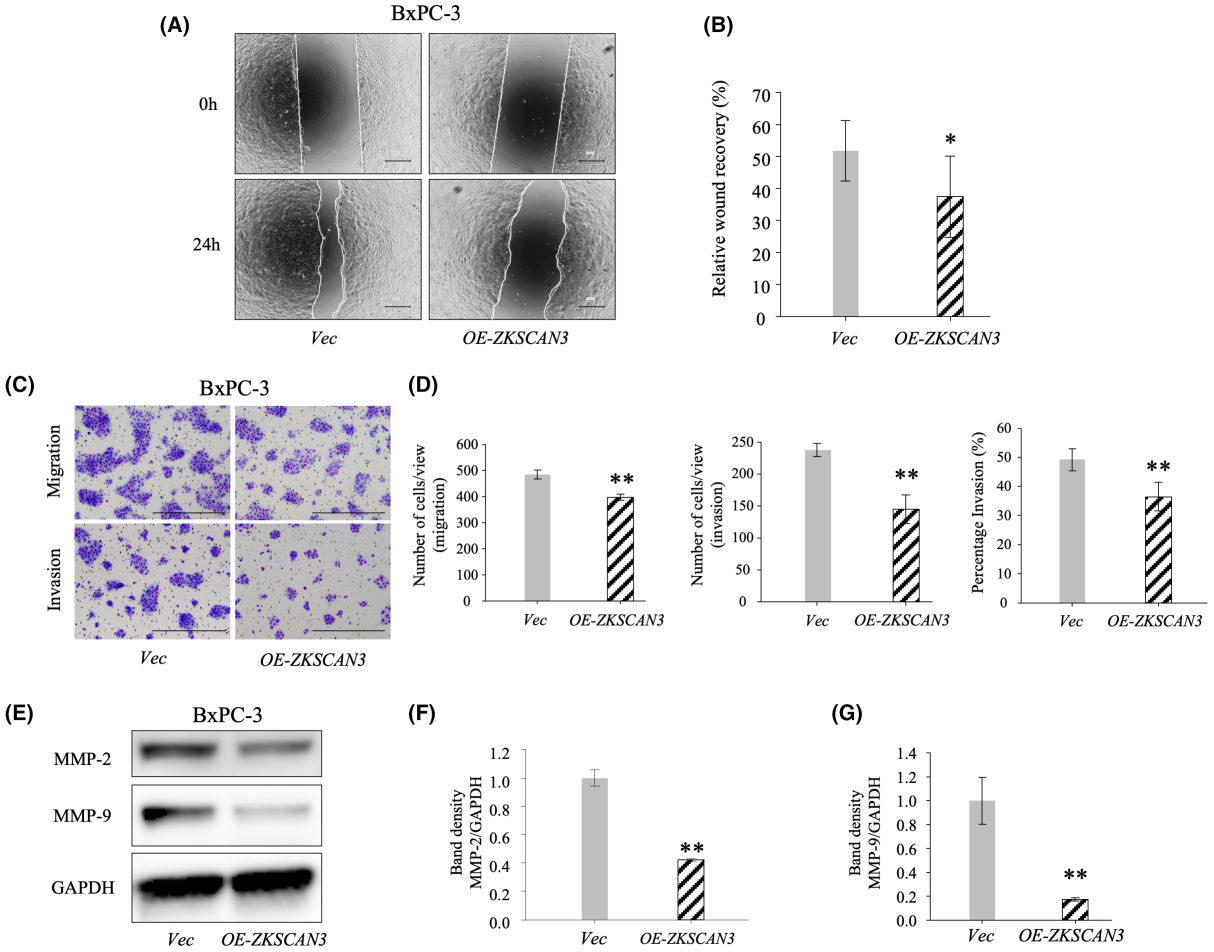

FIGURE 7 Changes in the migratory and invasive abilities, and matrix metalloproteinase expression in BxPC-3 cells after *ZKSCAN3* overexpression. (A) Wound healing assay. BxPC-3 cells ($1–2\times10^5$) were seeded in 24-well plates and cultured at 37°C for 24 h. When the cells reached at least 90% confluency, a sterile P200 pipette tip was used to create a straight scratch in the cell monolayer. The cells were cultured for 24 h in medium containing 2% FBS and were subsequently observed under a phase-contrast microscope. Scale bars, 500 μm. (B) The area of the wound was calculated, and the % of the wound area filled was determined using the following formula: % wound area filled = $(S_0 - S_a)/S_0 \times 100$. Here, S_0 represents the initial wound area, and Sa represents the wound area after culture. (C) The migratory and invasive abilities of BxPC-3 cells were assessed using the Boyden double chamber method. Scale bar, 500 μm. (D) The number of invading cells in nine random microscopic fields was quantified. Percentage invasion was calculated by dividing the number of invading cells through Matrigel-coated inserts by the number of cells invading through non-coated inserts. (E) Changes in the expression of MMP-2 and MMP-9 due to *ZKSCAN3* overexpression were evaluated using western blotting. (F, G) Band densities of MMP-2 and MMP-9 on western blots from each cell line were calculated relative to the band density of GAPDH. Comparisons between groups were assessed using unpaired Student's *t*-test. $^*p<0.05$; $^{**}p<0.01$.

4 | DISCUSSION

Autophagy is considered a potential therapeutic target in the treatment of PaCa. Clinical trials have investigated the use of autophagy inhibitors as a monotherapy or in combination with chemotherapy for the treatment of PaCa, but the efficacy has been limited, necessitating further research.[29–31] This study was designed to examine how a transcription factor that inhibits autophagy, ZKSCAN3, functions in PaCa cells and to determine whether controlling the expression of ZKSCAN3 could regulate the progression of PaCa cells. Our research revealed that the expression of *ZKSCAN3* was increased in several PaCa cell lines compared with HPDE cells. Using web databases, we confirmed that *ZKSCAN3* mRNA expression is higher in PaCa tissues than in normal pancreatic tissues. Furthermore, we confirmed that *ZKSCAN3* mRNA expression is inversely correlated with the prognosis of PaCa patients, meaning that strong expression of *ZKSCAN3* in PaCa patients is associated with extended OS and a favorable trend in RFS. Knocking down *ZKSCAN3* expression resulted in significantly enhanced proliferation, migration, and invasion by PaCa cells, whereas overexpression of *ZKSCAN3* significantly suppressed these processes. Furthermore, knocking down *ZKSCAN3* led to increased expression of LC3-II, whereas overexpression of *ZKSCAN3* reduced LC3-II expression. In a xenograft mouse model, we confirmed that knocking down *ZKSCAN3* resulted in tumor enlargement.

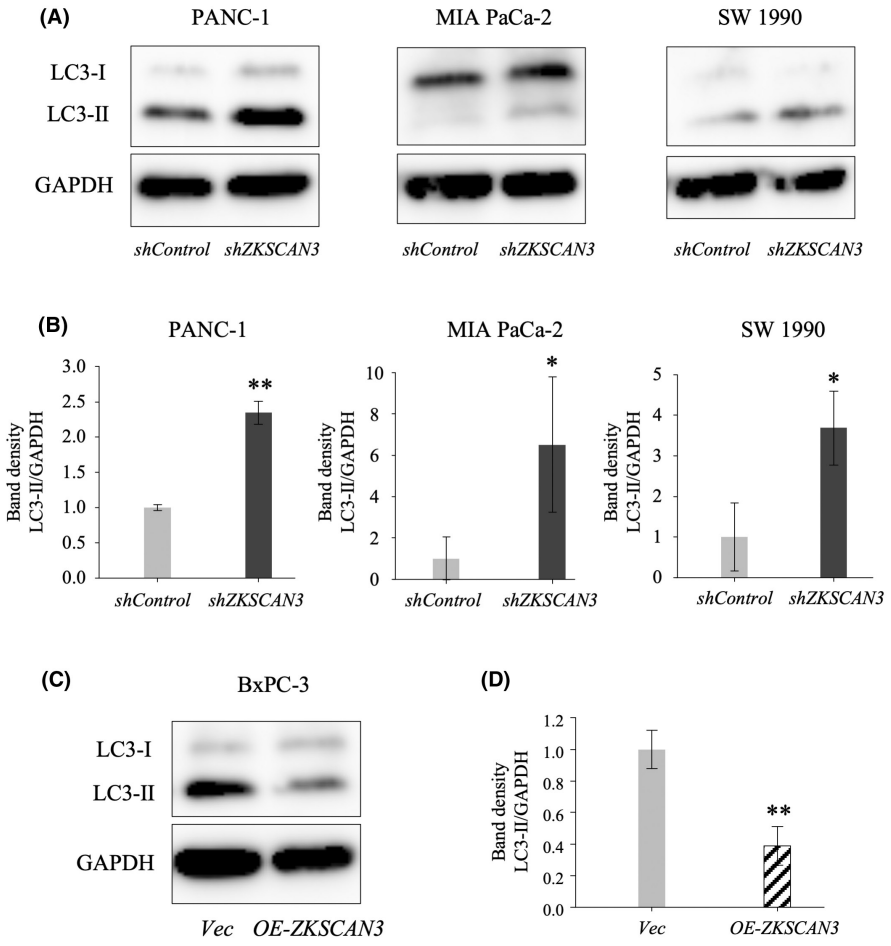

FIGURE 8 The changes in the expression of the autophagy marker LC3-II following the regulation of ZKSCAN3 expression. (A) After transfection with *ZKSCAN3* shRNA, the expression of LC3-II protein was evaluated using western blotting. (B) The band densities of LC3-II in western blots for each PaCa cell line were calculated relative to the band density of GAPDH. (C) The expression of LC3-II protein after transfection with the *ZKSCAN3* expression construct was evaluated using western blotting. (D) The band densities of LC3-II in western blotting for the BxPC-3 cell line were calculated relative to the band density of GAPDH. $^{*}p < 0.05$; $^{**}p < 0.01$.

The upregulation of *ZKSCAN3* expression has been observed in various tumors.[18–25] ZKSCAN3 is a transcription factor involved in various biological processes, including transcriptional suppression of autophagy, and alleviation of cellular senescence; it also contributes to tumorigenesis and cancer progression.[32,33] ZKSCAN3 was initially identified as a factor contributing to the malignancy of colorectal cancer.[18] Subsequently, in various other tumors such as prostate cancer, bladder cancer, and breast cancer, elevated expression of ZKSCAN3 has been reported to be associated with tumor formation, proliferation, migration and invasion.[20–22] Thus far, the relationship between PaCa and ZKSCAN3 has not been analyzed.

Here, we report for the first time that *ZKSCAN3* is overexpressed in multiple PaCa cell lines. Intriguingly, high expression of *ZKSCAN3* was significantly associated with extended OS+ in PaCa. Moreover, knocking down *ZKSCAN3* significantly promoted the progression of PaCa, while overexpressing *ZKSCAN3* inhibited the progression of PaCa. Furthermore, *ZKSCAN3* knockdown enhanced autophagic activity in PaCa, whereas overexpression of *ZKSCAN3* suppressed autophagic activity in PaCa. Zhang et al.[16] reported the involvement of *ZKSCAN3* in the regulation of autophagy in hepatocellular carcinoma, supporting the results of this study. *ZKSCAN3* also has been reported to be involved in the regulation of autophagy in both bladder cancer cells and colorectal cancer cells.[10] However, it has been suggested that knocking down *ZKSCAN3* in these cancer cells promotes autophagy, thereby promoting cellular senescence and inhibiting the growth of cancer cells.[10] In contrast, autophagy is essential for tumor growth in PaCa.[34] Additionally, PaCa cells exhibit higher levels of basal autophagy compared with breast cancer cells and lung cancer cells.[34] We speculate that the paradoxical function of *ZKSCAN3* arises from variations in the degree of dependence on autophagy, differences in the role of autophagy, and cell-specific contexts in each cancer cell.

In PANC-1 and MIA PaCa-2 cell lines, knocking down *ZKSCAN3* enhanced the invasive ability of PaCa cells. Additionally, in these two cell lines, knocking down *ZKSCAN3* resulted in an increase in the expression of MMP-2 and/or MMP-9. However, in SW 1990 cells in which *ZKSCAN3* was knocked down, there was no change in the invasive ability of PaCa cells. Furthermore, there was no change in the expression of MMP-9, whereas the expression of MMP-2 decreased. This suggests the potential involvement of other matrix MMPs. For example, in PaCa, MMP-1, MMP-2, MMP-7, MMP-9, membrane type 1 matrix metalloproteinase (MT1-MMP), membrane type 2 matrix metalloproteinase (MT2-MMP), and membrane type 3 matrix metalloproteinase (MT3-MMP) are expressed.[35,36] Moreover, MT1-MMP has been identified as an activator of MMP-2, directly involved in the degradation of the extracellular matrix. It is reportedly expressed from the early stages of tumor formation, with its expression levels

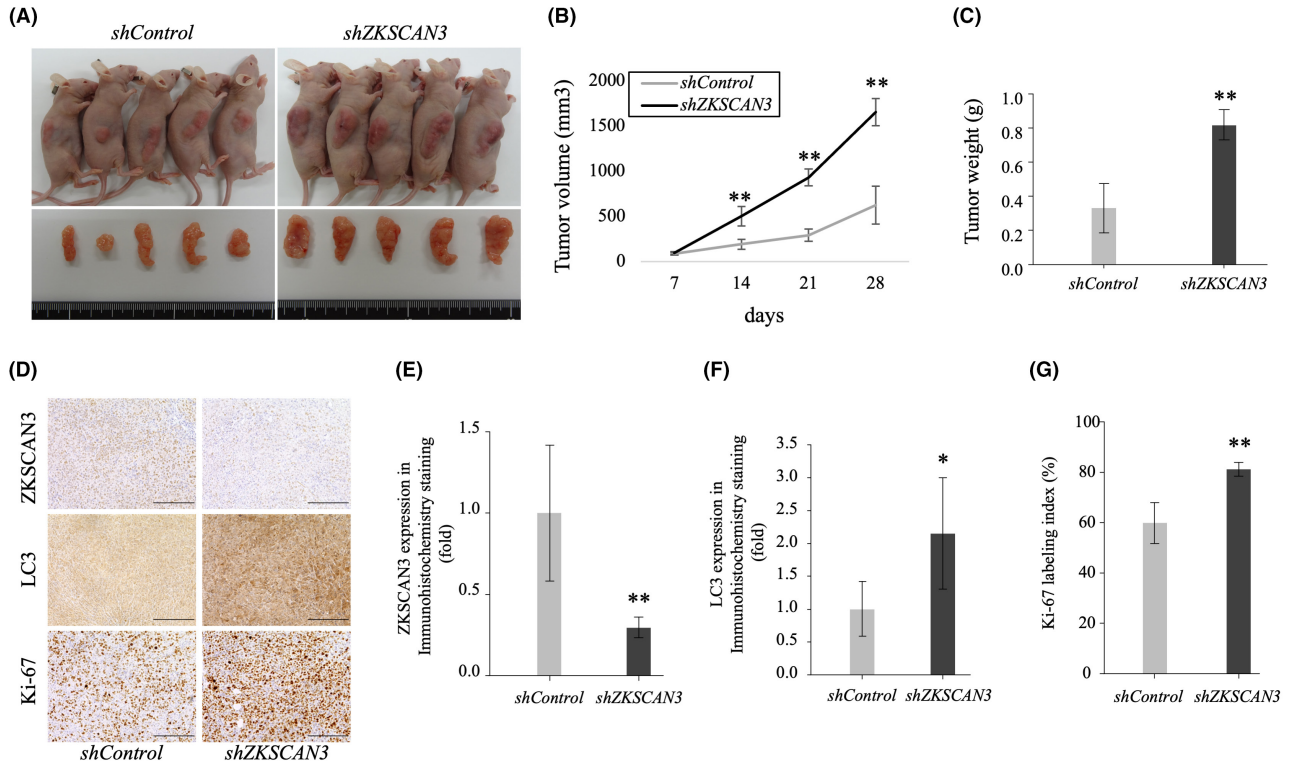

FIGURE 9 The knockdown of *ZKSCAN3* promoted tumor growth in a subcutaneous xenograft model of PaCa. Ten nude mice were divided into two groups of five. MIA PaCa-2 cells transfected with Control-shRNA or MIA PaCa-2 cells transfected with *ZKSCAN3*-shRNA (5×10^6 cells in 200 μL of Hank's balanced salt solution) were injected subcutaneously to grow tumors. (A) Images of mice and solid tumors extracted from each group. (B) Weekly measurements of tumor volumes in each group. (C) Measurements of tumor weights in sacrificed nude mice. (D) Representative immunohistochemical images (200×: ZKSCAN3, Ki-67 and LC3). Scale bars, 200 μm. (E) Comparison of immunohistochemical staining intensities of ZKSCAN3 between the two groups. (F) Comparison of immunohistochemical staining intensity of LC3 between the two groups. The immunohistochemical staining intensity of both ZKSCAN3 and LC3 was assessed relative to the staining intensity of the shControl group. (G) Comparison of the percentage of Ki-67 positive cells between two groups. $^*p<0.05$; $^{**}p<0.01$.

correlating with malignancy.[37,38] In SW 1990 cells, despite the decrease in MMP-2 expression following *ZKSCAN3* knockdown, the percentage invasion did not decrease. Further research is necessary to understand this phenomenon. Specifically, a comprehensive analysis of MMP expression in PaCa due to *ZKSCAN3* knockdown is required.

There are several limitations in this study. First, while this study confirmed the relationship between *ZKSCAN3* mRNA expression and the prognosis of PaCa, it did not investigate the relationship between ZKSCAN3 protein expression and PaCa prognosis. Furthermore, more detailed studies are necessary for SW 1990 cells to elucidate why invasion did not change despite the decrease in MMP-2 expression following *ZKSCAN3* knockdown. Thus, the expression of other MMPs should be determined. Additionally, we have not been able to confirm changes in the expression of proteins related to autophagy other than LC3. Thus, detailed signal transduction changes should be characterized when altering the expression of ZKSCAN3 in PaCa. These aspects require comprehensive analysis and will be addressed in future research.

Enhancing ZKSCAN3 expression suppresses autophagy in PaCa and inhibits its progression. Utilizing ZKSCAN3 expression enhancement as monotherapy or in combination with other chemotherapies may present a more effective treatment strategy for PaCa. However, there are several potential challenges to targeting ZKSCAN3 for clinical purposes. First, the upstream transcription factors and natural products that regulate the expression of ZKSCAN3 are not fully elucidated. Zhang et al.[16] reported that the *CHD1L* gene regulates *ZKSCAN3* transcription in hepatocellular carcinoma, but its mechanism in PaCa remains unknown. Additionally, a method to selectively control ZKSCAN3 expression in PaCa in vivo has yet to be elucidated. We plan to clarify these research challenges in the future.

In conclusion, this study is the first to elucidate the biological role of *ZKSCAN3* in PaCa. The results have revealed the increased expression of *ZKSCAN3* in several PaCa cell lines. Furthermore, it has been demonstrated that controlling *ZKSCAN3* expression can regulate the progression of PaCa through autophagy. Therefore, the regulation of autophagy via ZKSCAN3 suggests a potential novel therapeutic target for PaCa. In future research, the development of methods to overexpress ZKSCAN3 in PaCa and exploration of combination therapies including autophagy modulators are expected.

AUTHOR CONTRIBUTIONS

Keisuke Nonoyama: Conceptualization; data curation; formal analysis; writing – original draft; writing – review and editing. **Yoichi Matsuo:** Conceptualization; supervision. **Saburo Sugita:** Investigation. **Yuki Eguchi:** Investigation. **Yuki Denda:** Investigation. **Hiromichi Murase:** Investigation. **Tomokatsu Kato:** Data curation. **Hiroyuki Imafuji:** Data curation. **Kenta Saito:** Methodology. **Mamoru Morimoto:** Methodology. **Ryo Ogawa:** Investigation. **Hiroki Takahashi:** Investigation. **Akira Mitsui:** Methodology. **Masahiro Kimura:** Methodology. **Shuji Takiguchi:** Supervision.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest.

ETHICS STATEMENTS

Approval of the research protocol by an Institutional Reviewer Board: N/A.

Informed Consent: N/A.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: N/A.

ORCID

Yoichi Matsuo https://orcid.org/0000-0001-9654-6080

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