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Exosomal zinc transporter ZIP4 promotes cancer growth and is a novel diagnostic biomarker for pancreatic cancer

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Pancreatic cancer is one of the deadliest cancers with rapid disease progression. Further elucidation of its underlying molecular mechanisms and novel biomarkers for early detection is necessary. Exosomes are small extracellular vesicles that are released by multiple cell types acting as message carriers during intercellular communication and are promising biomarker candidates. However, the role of pancreatic cancer cell-derived exosomes in cancer progression and the application of these vesicles as novel diagnostic biomarkers have not been fully studied. In this study, we found that PC-1.0 (a highly malignant pancreatic cell line) cell-derived exosomes could be taken up by and enhance PC-1 (a moderately malignant pancreatic cell line) cell proliferation, migration and invasion abilities. We identified ZIP4 as the most upregulated exosomal protein in PC-1.0 cells from our proteomic analysis. In vitro and in vivo (a subcutaneous BALB/c nude mouse model) studies showed that exosomal ZIP4 can significantly promote pancreatic cancer growth. Using clinical blood samples, we compared the diagnostic values of serum exosomal ZIP4 levels between malignant pancreatic cancer patients (n = 24) and benign pancreatic disease patients (n = 32, AUC = .89), and between biliary disease patients (n = 32, AUC = .8112) and healthy controls (n = 46, AUC = .8931). In conclusion, exosomal ZIP4 promotes cancer growth and is a novel diagnostic biomarker for pancreatic cancer.

KEYWORDS
biomarker, exosomes, pancreatic cancer, proteomics, zinc transporter ZIP4

1 | INTRODUCTION

Pancreatic cancer is one of the deadliest cancers and is ranked fourth in cancer-related mortality.[1] Patients with pancreatic cancer are often diagnosed at an advanced stage due to the rapid progression and poor early detection rate of this cancer type. Usually, only 10%-20% of the patients at this stage are considered for surgery, which remains the only curative treatment option.[2,3] However, pancreatic cancer patients are rarely cured by surgical resection due to the extensive local invasion and early distant metastatic potential of the disease. Thus, elevating the overall survival rate of pancreatic cancer relies on 2 aspects. For suspected patients or a high-risk population, it is more critical to increase the early detection rate. For patients with a definite diagnosis, it is more urgent to suppress further progression. Both of the aforementioned aspects require further elucidation of the underlying molecular mechanisms of the disease.

Previously, we used 2 isogenic pancreatic cell lines, PC-1.0 (highly malignant) and PC-1 (moderately malignant), to study the molecular mechanism of pancreatic cancer progression.[4-8] We found that secretory proteins from the conditioned medium of PC-1.0 cells could enhance the proliferation and metastatic abilities of PC-1 cells, and these proteins were later named dissociation factors (DF).[9]

Aiming to uncover the nature of DF, we performed a quantitative secretomic analysis of the conditioned media of these 2 cell lines.[10] It is noteworthy that some secretory proteins are secreted as the cargo of extracellular vesicles. Exosomes are small (30-100 nm) extracellular vesicles derived from multivesicular bodies containing and transporting cargos, such as proteins, noncoding RNAs and DNAs,[11] and are released by multiple cell types acting as message carriers during intercellular communication.[12,13] Exosomes have been reported to play multiple roles during cancer progression, such as the formation of a premetastatic niche, determination of organotropic metastasis, promotion of epithelial-mesenchymal transition, regulation of the local microenvironment, and transportation of oncogenic proteins.[14-21] Exosomal proteins are more stable and detectable than regular secretory proteins in body fluids, which makes them perfect candidates as novel biomarkers.[22-24] Combining our previous studies and the reported functions of exosomes, we hypothesize that the exosomes from PC-1.0 cells are more enriched in key proteins promoting cancer progression and could transport these oncogenic proteins to PC-1 cells. Furthermore, the enriched exosomal proteins of PC-1.0 may serve as novel biomarkers for pancreatic cancer.

In this study, utilizing in vitro cellular assays, we showed that PC-1.0-derived exosomes could be taken up and enhance the proliferation, migration and invasion abilities of PC-1 cells. Proteomic and bioinformatic analyses of the differential exosomal proteins between PC-1.0 and PC-1 showed that PC-1.0-derived exosomes were more enriched in proteins that play key roles in cancer progression. The zinc transporter protein ZIP4 was the most upregulated protein in PC-1.0-derived exosomes. ZIP4 is a membrane-located (plasma membrane, endosome membrane and extracellular vesicle membrane) zinc ion transporter regulating intracellular zinc homeostasis. ZIP4 is reported to be differentially expressed in multiple cancers and to be closely related to the progression of cancer, including pancreatic cancer.[25–33] In vitro and in vivo studies have shown that exosomal ZIP4 can significantly promote pancreatic cancer growth. Using clinical blood samples from patients with malignant pancreatic cancer, patients with benign pancreatic disease, patients with biliary disease and healthy subjects, for the first time, we showed the efficacy of exosomal ZIP4 as a novel diagnostic biomarker for pancreatic cancer.

2 | MATERIALS AND METHODS

2.1 | Ethics approval and consent to participate

This study was approved by the local ethics committee of the Affiliated Shengjing Hospital of China Medical University with certificate numbers 2017PS006K for the animal studies and 2017PS24K for the blood samples. All animal handling procedures were performed in accordance with the standard of the Ethics Committee of Shengjing Hospital. Written informed consent including all the necessary details of this study was provided by all participants for the collection of blood samples at the time of initial admission.

2.2 | Cell lines and cell culture

Two hamster pancreatic cancer cell lines were used as follows: PC-1.0 (highly malignant) and PC-1 (moderately malignant). The PC-1 cell line was established from pancreatic ductal adenocarcinomas induced by (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluophosphate (BOP) in a Syrian golden hamster. The PC-1.0 cell line was established from a subcutaneous tumor produced after the inoculation of a hamster with PC-1 cells.[34] The human pancreatic cancer cell lines AsPC-1 and Capan-2 were purchased from the American Tissue Culture Collection (Rockville, MD, USA). Cells were incubated as previously described.[10]

2.3 | Exosome isolation from cell culture supernatant

Exosomes were isolated from the supernatants of cultured cells with an SBI ExoQuick-TC Kit (System Biosciences, Mountain View, CA, USA) according to the manufacturer's protocol with minor modifications. The supernatants were purified in advance using Amicon Ultra15 100K ultrafilter devices (Millipore, Billerica, MA, USA) to remove nonexosomal proteins. For proteomic analysis and electron microscopy, the initial pellet was resuspended in sterile PBS. The resuspended pellet was isolated again with an ExoQuick-Tc Kit using the same procedure to further remove nonexosomal proteins.

2.4 | Electron microscopy

The sample preparation was performed as described previously.[35,36] In brief, isolated exosome pellets were fixed with 2.5% glutaraldehyde and washed twice with PBS. The pellets were then stained with aqueous phosphotungstic acid and fixed on copper mesh Formvar grids. The samples were observed with a HITACHI H-7650 transmission electron microscope (HITACHI, Tokyo, Japan).

2.5 | Western blot assay

Western blotting was performed as described previously.[4] Samples of equivalent total protein (20 μg) were loaded. Primary antibodies against CD63, CD81, HSP70 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-Actin, ZIP4, Adiponectin, MMP9 and MMP12 (Protein-Tech Group, Rosemont, IL, USA) were used.

2.6 | Fluorescent labeling of exosomes

The exosomal proteins were labeled fluorescent green with Exo-Glow (System Biosciences) according to the manufacturer's protocol. Further consecutive visualization with fluorescence microscopy was performed for 24 hours.

2.7 | Cell proliferation assay

Cell proliferation assays were performed using a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Tokyo, Japan) according to

the manufacturer's protocol. Exosomes equaling 500 ng of exosomal proteins were added per well if needed.

2.8 | In vitro migration assay

Wound-healing migration assays were performed as described in our previous studies.[10] Exosomes equaling 10 μg of exosomal proteins were added to the culture medium if needed.

2.9 | In vitro invasion assay

Invasion assays using the Transwell system (24 wells, 8-μm pore size filters, Costar, New York, USA) were performed as described in our previous studies.[10] Exosomes equaling 5 μg of exosomal proteins were added to the upper chamber together with the cells if needed.

2.10 | Protein digestion and isobaric tags for relative and absolute quantification labeling

Protein digestion was performed according to the standard procedure, and the resulting peptide mixture was labeled using 4-plex or 8-plex isobaric tags for relative and absolute quantification (iTRAQ) reagent according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The samples were labeled (PC-1)-119 and (PC-1.0)-121.

2.11 | High pH reverse-phase liquid chromatography separation

The samples were fractionated using high pH reversed-phase separation to increase proteomic depth. The peptides were resuspended in the loading buffer (5 mmol/L ammonium formate containing 2% acetonitrile, pH 10) and separated by high pH reversed-phase liquid chromatography (RPLC, Acquity Ultra Performance LC, Waters, Milford, USA). The gradient elution was performed on a high pH RPLC column (C18, 3.5 μm, 150 × 2.1 mm, Waters) at 200 μL/min with the gradient increasing for 60 minutes. Twenty fractions were collected from each sample and were subsequently pooled, resulting in 10 total fractions per sample.

2.12 | Liquid chromatography tandem mass spectrometry

The experiments were performed on a Q Exactive mass spectrometer, which was coupled to the Easy-nLC system. Ten microliters of each fraction were injected for nanoLC-MS/MS analysis. The peptide mixture (2 μg) was loaded onto a C18-Reversed-Phase Column (75 μm × 25 cm, Thermo Fisher Scientific, Waltham, MA, USA) in buffer A (.1% formic acid) and separated with a linear gradient of buffer B (80% acetonitrile and .1% formic acid) at a flow rate of 300 nL/min. An electrospray voltage of 1.9 kV versus the inlet of the mass spectrometer was used. A Q Exactive Mass Spectrometer was operated in data-dependent mode to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra (m/z 300-1200) were acquired with a mass resolution of 70 k followed by 15 sequential high-energy collisional dissociation MS/MS scans with a resolution of 17.5 k. In all cases, 1 microscan was recorded using dynamic exclusion of 30 seconds. For MS/MS, precursor ions were activated using 27% normalized collision energy.

2.13 | Data analysis

MS/MS spectra were searched using Proteome Discoverer Software 2.1 (Thermo Fisher Scientific) against the UniProt database. The highest score for a given peptide mass (best match to that predicted in the database) was used to identify the parent proteins. The parameters for protein searching were set as follows: tryptic digestion with up to 2 missed cleavages, carbamidomethylation of cysteines as a fixed modification, oxidation of methionines and protein N-terminal acetylation as variable modifications. Peptide spectral matches were validated based on the *q*-values at a 1% false discovery rate (FDR). Protein abundance ratios with at least 1.5-fold change were considered differentially expressed proteins (DEP).

2.14 | Exosomal ZIP4 uptake assay

PC-1.0-derived exosomes equaling 20, 40 and 60 μg exosomal proteins were added to the supernatant of a normal T25 cell culture flask containing PC-1 cells at 60%-70% confluency. The ZIP4 protein level of the PC-1 cells was tested by western blot analysis after another 24 hours of culture. Normal PC-1 cells under the same culture conditions were used as the control.

2.15 | shRNA design and plasmid transfection

The shRNA plasmids specific for ZIP4 were purchased from GeneChem (Shanghai, China). Three different shRNA against ZIP4 were designed with the target sequences: Slc39a4-RNAi (5437-1): 5′-GTCCAAACAGACCCATGAA-3′; Slc39a4-RNAi (5438-1): 5′-TCCCA ATATCACGCTGCAT-3′; and Slc39a4-RNAi (5439-1): 5′-TGGAGTC-CAGACACATTAT-3′. The shRNA plasmids were transfected into the PC-1.0 cells using Lipofiter Liposomal Transfection Reagent (Hanbio Biotechnology, Shanghai, China). The infection efficiency was measured by GFP expression in transfected cells, and shRNA efficiency was measured by western blot.

2.16 | Cell cycle assay by flow cytometry

Cell cycle analysis was performed using a detection kit (Nanjing KeyGen Biotechnology, Nanjing, China) according to the manufacturer's protocol. Exosomes equaling 10 μg of exosomal proteins per well were added if needed.

2.17 | Animal studies

Subcutaneous tumor models were generated using 4-week-old female BALB/c nude mice (Huafukang Biotechnology, Beijing, China). The mice were kept and fed in a specific-pathogen-free level animal

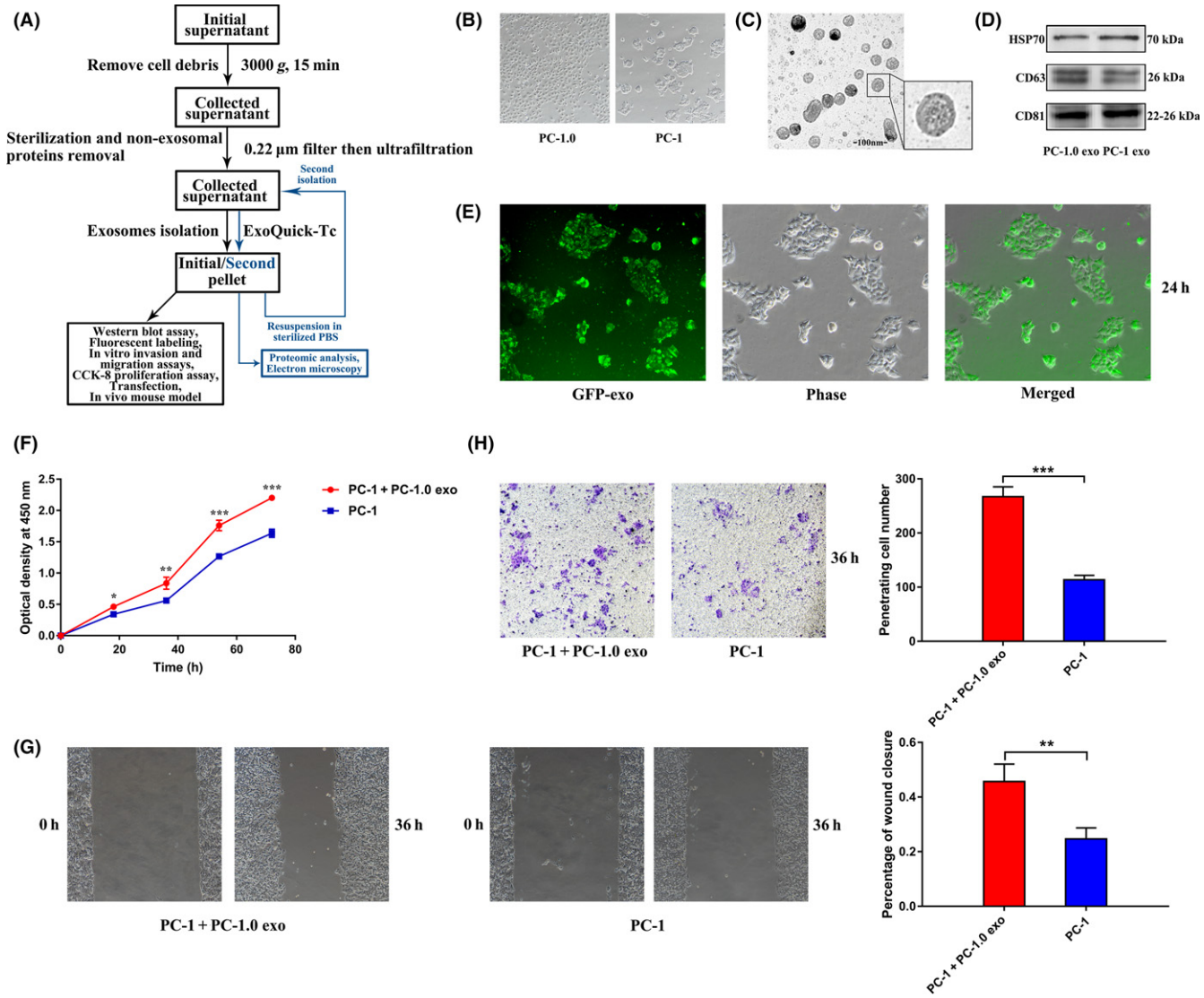


FIGURE 1 PC-1.0-derived exosomes could be taken up and enhance PC-1 cell proliferation, migration and invasion abilities. A, Flowchart of exosome isolation procedures in this study. B, Phase contrast images of PC-1.0 and PC-1 cells and their characteristic morphology. C, Representative transmission electron microscope image of exosomes isolated from PC-1.0 and PC-1 cell lines. D, Western blot validation of exosomal markers (HSP70, CD63 and CD81) of PC-1.0-derived and PC-1-derived exosomes. E, Representative fluorescence microscope images of PC-1.0-derived exosomes taken up by PC-1 cells. F, CCK-8 cell proliferation assays of PC-1 cells cocultured with PC-1.0-derived exosomes and PC-1 cells alone. G, Wound-healing migration assays of PC-1 cells cocultured with PC-1.0-derived exosomes and PC-1 cells alone (magnification, ×100). H, Transwell invasion assay of PC-1 cells cocultured with PC-1.0-derived exosomes and PC-1 cells alone (magnification, ×40). *$P < .05$; **$P < .01$; ***$P < .001$

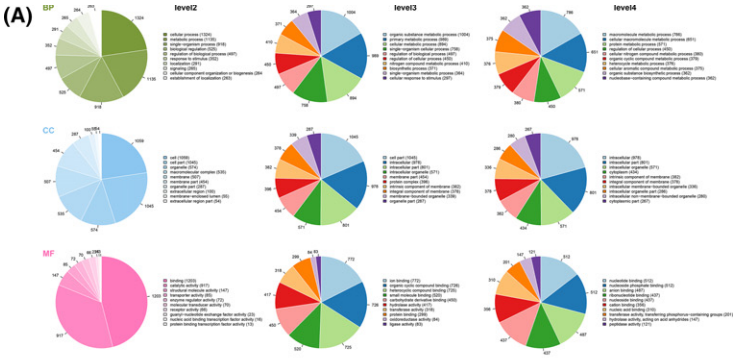
laboratory of Shengjing Hospital. A total of 1×10^6 PC-1 cells in .1-mL PBS were subcutaneously injected into the right axilla area. Ten days after the initial implantation, when the subcutaneous tumors were visible, the mice were injected with 60-μL PBS containing 30-μg normal PC-1.0-derived exosomes or ZIP4-knockdown exosomes in the implantation area twice a week. The size of the subcutaneous tumor was recorded twice a week with a Vernier caliper. The volume of the tumor was calculated using the following equation: tumor volume = $(L \times W^2)/2$, where L = tumor long axis and W = tumor short axis. All the measurements were repeated 3 times. Four weeks from the initial implantation, the mice were killed, and the tumor tissues were surgically excised and measured.

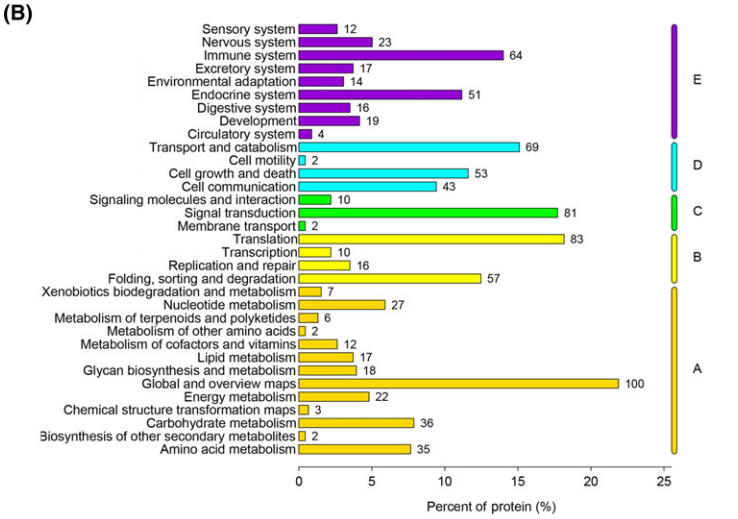
2.18 | Immunohistochemical assay

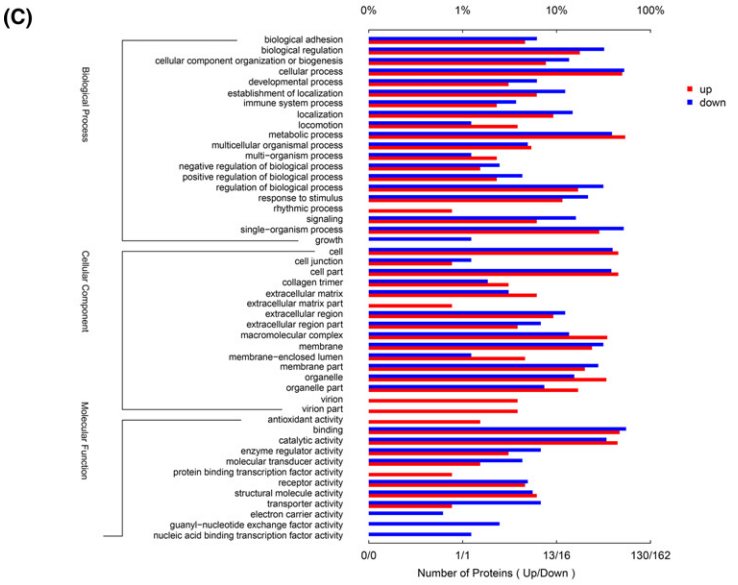
Immunohistochemical staining of the tumor tissue was performed according to the kit manufacturer's protocol (Zhongshan Golden Bridge, Beijing, China) on 4% paraformaldehyde-fixed, paraffin-embedded 4-μm tissue sections, which were later evaluated by 2 different specialists.

2.19 | Exosome isolation from human serum

For human serum samples, whole blood samples were collected from clinical routine blood tests and were stored for 1 hour at 4°C to be







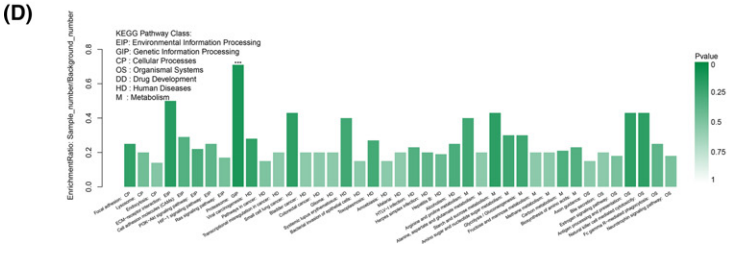


FIGURE 2 Bioinformatic analysis of whole and differentially expressed exosomal proteins from PC-1.0 and PC-1 cells. A, Gene ontology (GO) analysis of whole exosomal proteins. B, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of whole exosomal proteins. C, GO analysis of differential exosomal proteins (protein abundance ratios with at least 1.5-fold change). D, KEGG analysis of differentially expressed exosomal proteins. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$

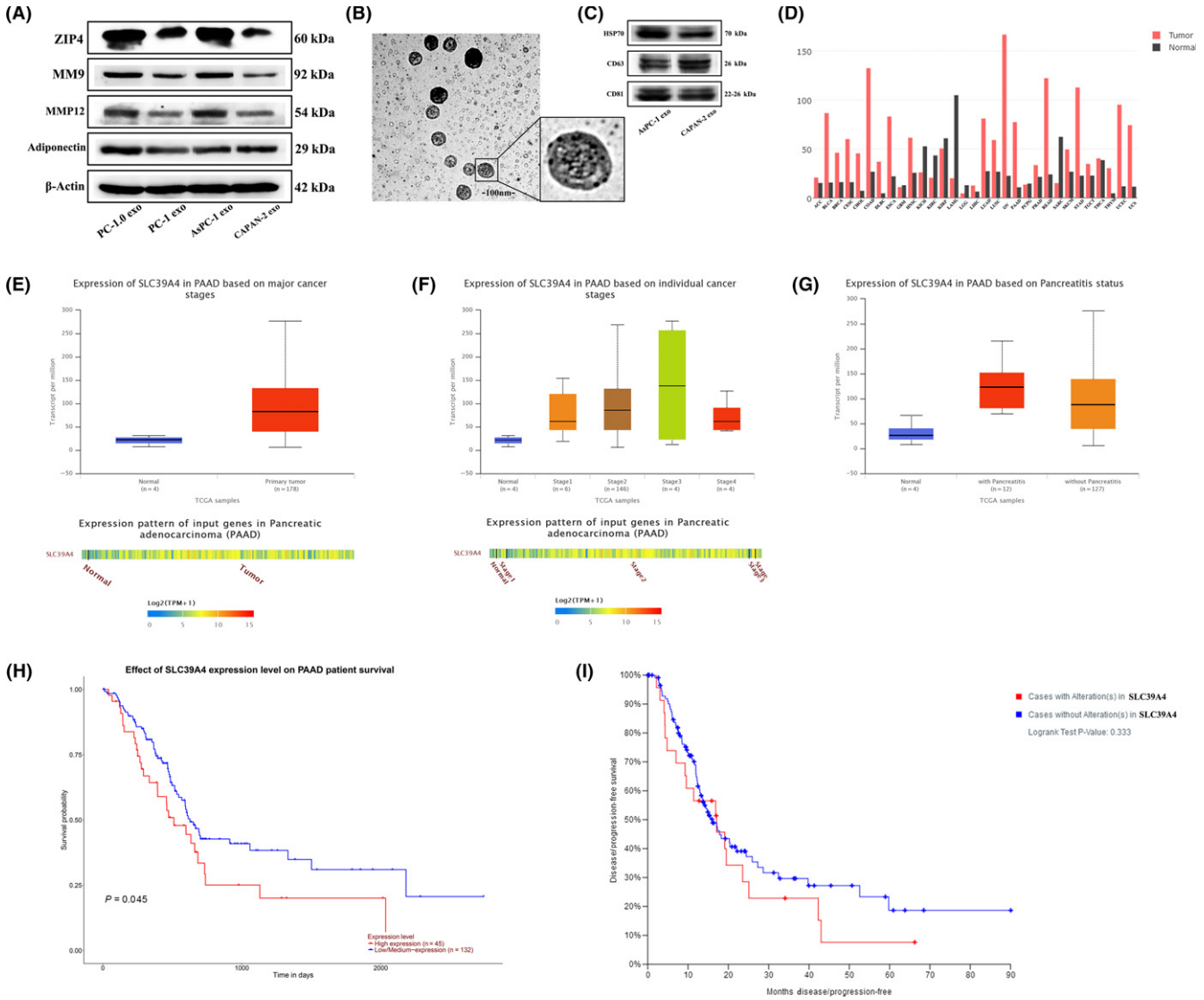


FIGURE 3 Exosomal ZIP4 is a potential regulator and novel biomarker for pancreatic cancer. A, Western blot validation of zinc transporter ZIP4, matrix metalloproteinase-9 (MMP-9), macrophage metalloelastase (MMP-12) and adiponectin with exosomes from PC-1.0, PC-1, AsPC-1 and Capan-2 cell lines. B, Representative transmission electron microscope images of AsPC-1-derived and Capan-2-derived exosomes. C, Western blot validation of exosomal markers (HSP70, CD63 and CD81) of AsPC-1-derived and Capan-2-derived exosomes. D, Expression levels of SLC39A4 (a gene encoding ZIP4 in humans) in multiple cancer types, as indicated by the TCGA database. E, SLC39A4 is differentially expressed in pancreatic cancer patients and healthy individuals. F, SLC39A4 is differentially expressed in patients with different stages of pancreatic cancer, as indicated by the TCGA database. G, SLC39A4 is differentially expressed in pancreatic cancer patients with different statuses of pancreatitis, as indicated by the TCGA database. H, Higher expression levels of SLC39A4 indicate poor survival rate in pancreatic cancer patients, as indicated by the TCGA database. I, Alterations in SLC39A4 indicate less progression/disease-free time in pancreatic cancer patients, as indicated by the TCGA database

fully coagulated before being centrifuged at 2600 *g* at 4°C for 10 minutes. The supernatants were collected and stored in a −80°C freezer for a maximum of 6 months. The exosomes were isolated from human serum samples with an ExoQuick Exosome Precipitation Kit (System Biosciences) according to the manufacturer's protocol.

2.20 | Enzyme-linked immunosorbent assay

The ZIP4 protein level of serum-derived exosomes was tested using a human ZIP4 ELISA kit (Cloud-Clone, Wuhan, China) according to the manufacturer's protocol. One hundred microliters of exosome samples (isolated from 250 μL of serum samples and resuspended in 200-μL PBS) was analyzed, and the protein concentration was calculated in comparison with a protein standard curve.

2.21 | Bioinformatic and statistical analyses

The following publicly available databases were utilized for the bioinformatics analysis: Gene Ontology (http://geneontology.org/), Kyoto Encyclopedia of Genes and Genomes (http://www.kegg.jp/kegg/pa

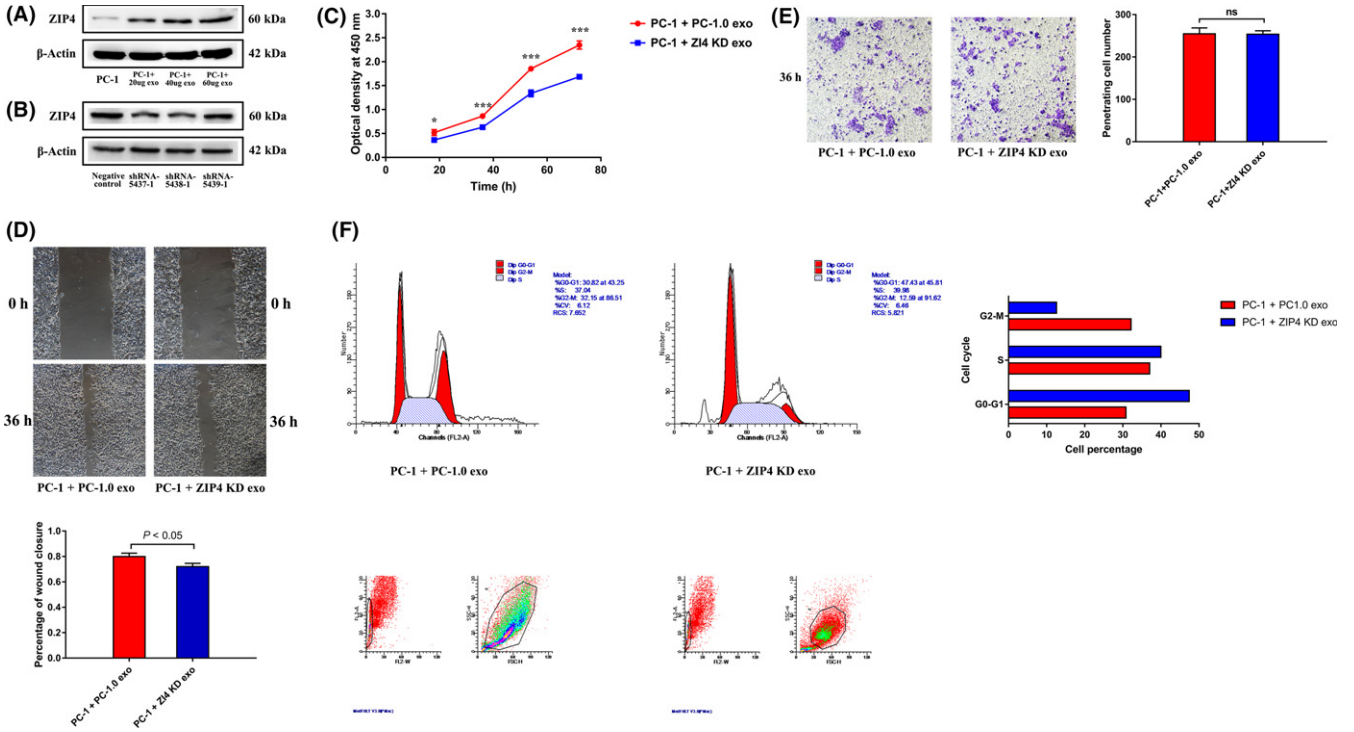


FIGURE 4 Exosomal ZIP4 promotes pancreatic cancer cell growth in vitro. A, The expression level of ZIP4 in PC-1 cells increased with the increase of cocultured PC-1.0-derived exosomes. B, Exosomal ZIP4 knockdown (KD) by shRNA transfection. C, CCK-8 cell proliferation assay of PC-1 cells cocultured with PC-1.0-derived normal and ZIP4-KD exosomes. D, Wound-healing migration assay of PC-1 cells cocultured with PC-1.0-derived normal and ZIP4-KD exosomes (magnification, ×100). E, Transwell invasion assay of PC-1 cells cocultured with PC-1.0-derived normal and ZIP4-KD exosomes (magnification, ×40). F, Cell cycle analysis of PC-1 cells cocultured with PC-1.0-derived normal and ZIP4 KD exosomes. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; ns, not significant

thway.html), Eukaryotic Orthologous Group (http://www.ncbi.nlm.nih.gov/COG/), cBioPortal (http://www.cbioportal.org/), UniProt (http://www.uniprot.org/), The Cancer Genome Atlas (https://cancergenome.nih.gov/), Gene Expression Profiling Interactive Analysis (http://gepia.cancer-pku.cn/) and UALCAN (http://ualcan.path.uab.edu/index.html). The wound closure distance was quantified using Adobe Photoshop CS6 (64 Bit) software, and the number of invaded cells was quantified using ImageJ software. Student's *t*-test was performed using IBM SPSS Statistics version 19. *P*-values < .05 were considered statistically significant. The statistical charts were designed with GraphPad Prism 7.

3 | RESULTS

3.1 | PC-1.0-derived exosomes could be taken up and enhance PC-1 cell proliferation, migration and invasion abilities

According to the purpose of the experiments in this study, we applied different exosome isolation procedures (Figure 1A). In the in vitro culturing environment, the PC-1.0 cells mainly grew as single cells with a doubling time of 13 hours, while the PC-1 cells grew in an island-like formation with a doubling time of 39 hours (Figure 1B).[34] Transmission electron microscopy was used to visualize the purified exosomes from these 2 cell lines. Representative images show that the purified exosomes were mainly round-shaped vesicles ranging from 50 to 100 nm with a clear membrane structure (Figure 1C). Western blot analysis validated the expression of the known exosomal biomarkers CD63, CD81,and HSP70 (Figure 1D). The exosomal proteins of the PC-1.0 cells were labeled fluorescent green and coincubated with the PC-1 cells. Fluorescence microscopy was used to confirm the uptake of PC-1.0-derived exosomes by the PC-1 cells (Figure 1E). A CCK-8 cell proliferation assay, wound-healing migration assay and Transwell invasion assay were further performed to investigate the effects of PC-1.0-derived exosomes on PC-1 cells. The results showed that PC-1.0-derived exosomes significantly enhanced the proliferation, migration and invasion abilities of cocultured PC-1 cells (Figure 1F-H).

3.2 | Proteomic and bioinformatic analyses of PC-1.0-derived and PC-1-derived exosomes

Isobaric tags for relative and absolute quantification (iTRAQ)-based proteomic analysis of PC-1.0-derived and PC-1-derived exosomes identified a total of 2707 proteins, and of these proteins, 154 proteins were upregulated and 189 proteins were downregulated in PC-1.0-derived exosomes (fold change >1.5 or fold change <.67) (Supplementary Table S1). Gene ontology (GO) analysis, Eukaryotic Orthologous Group (KOG) function classification and Kyoto Encyclopedia of Genes and

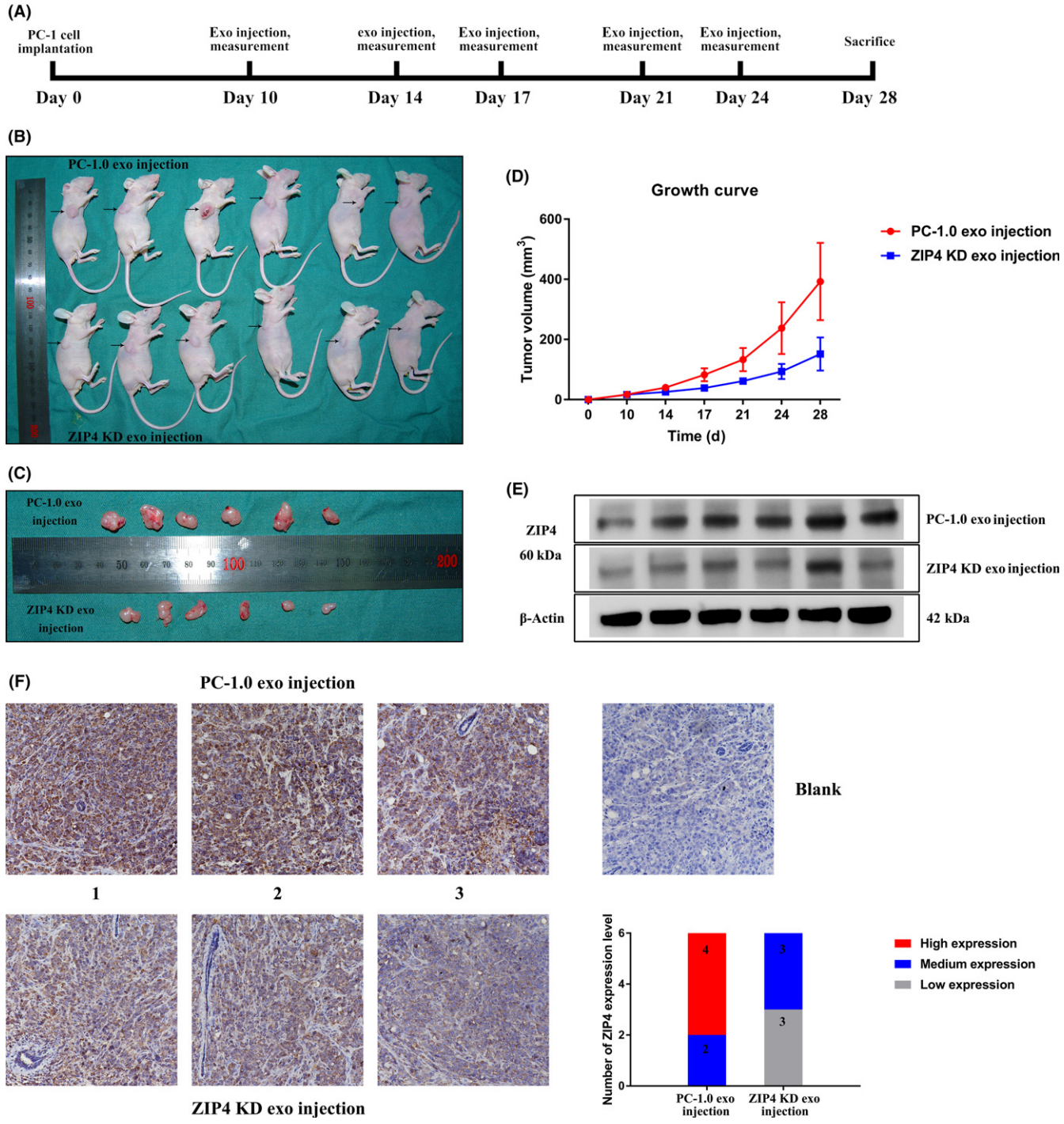


FIGURE 5 Exosomal ZIP4 promotes pancreatic cancer growth in a nude mouse model. A, Experimental design of the animal studies. B, Subcutaneously implanted tumor 4 wk after initial implantation. C, Surgically excised tumor tissue 4 wk after initial implantation. D, Growth curve of subcutaneously implanted tumors. E, Western blot analysis of ZIP4 levels in the excised tumor tissue. F, Representative images of immunohistochemical staining of ZIP4 in a paraffin-embedded excised tumor tissue section (magnification, ×200). $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$

Genomes (KEGG) pathway analysis were performed to analyze the functional and cellular component distributions and the enriched pathways of the whole exosomal proteins (Figure 2A,B, Supplementary Figure S1A).[37-39] The top 20 enriched pathways could be mainly divided into endocytosis, signaling pathways in cancer, RNA processing and transport, focal adhesion and cellular junction, which were consistent with known functions of cancer-derived exosomes (Supplementary Figure S1B). A heat map was utilized to depict all the differential exosomal proteins between PC-1.0 and PC-1 cells (Supplementary Figure S2). Furthermore, GO and KEGG pathway analyses were performed to

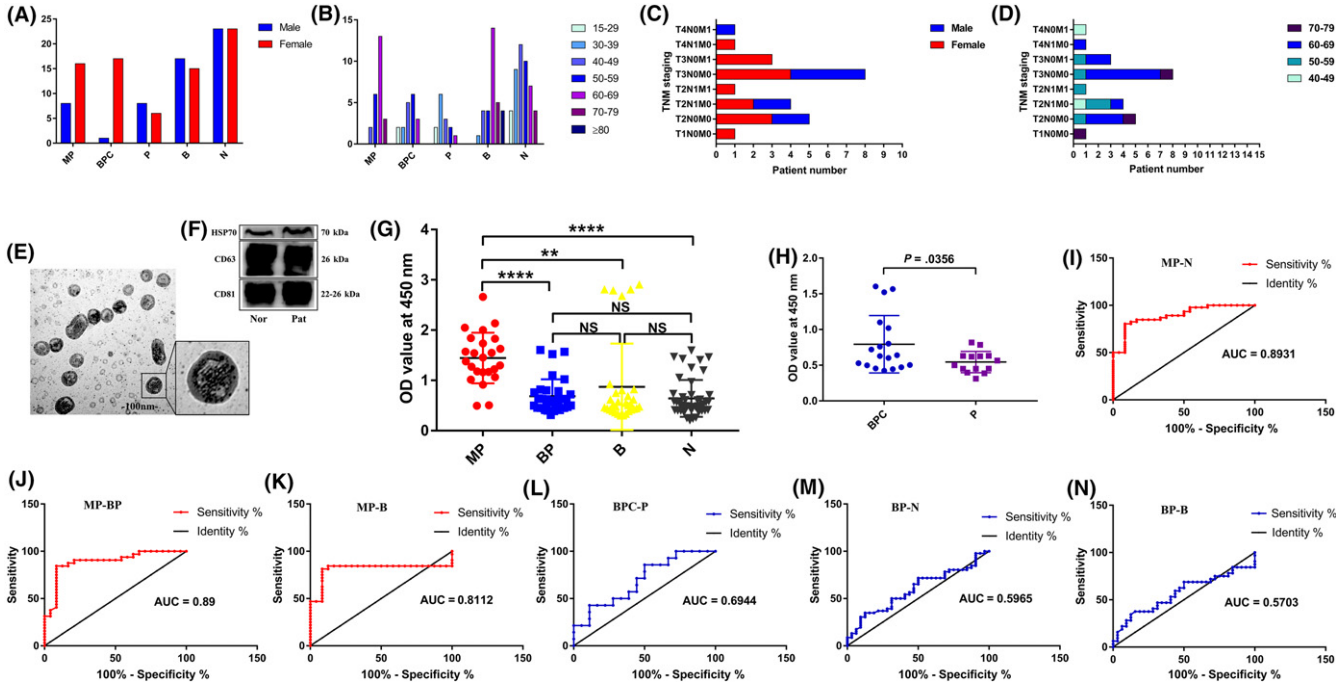


FIGURE 6 Exosomal ZIP4 is a novel diagnostic biomarker for pancreatic cancer. A, Bar chart of the gender distribution of the clinical participants. B, Bar chart of the age distribution of the clinical participants. C, Bar chart TNM staging distribution of the genders of the pancreatic cancer patients. D, Bar chart TNM staging distribution of the ages of the pancreatic cancer patients. E, Representative transmission electron microscope image of exosomes isolated from clinical serum samples. F, Western blot validation of exosomal markers (HSP70, CD63 and CD81) of exosomes isolated from clinical serum samples. G, Exosomal ZIP4 level distribution in clinical serum samples. MP, malignant pancreatic cancer; BP, benign pancreatic diseases; BPC, benign pancreatic tumors; P, pancreatitis; B, biliary disease; N, normal controls. H, Exosomal ZIP4 level distribution between BPC and P groups. I, Receiver operating characteristic curve (ROC) curve analysis of exosomal ZIP4 between MP and N groups. J, ROC curve analysis of exosomal ZIP4 between MP and BP groups. K, ROC curve analysis of exosomal ZIP4 between MP and B groups. L, ROC curve analysis of exosomal ZIP4 between BPC and P groups. M, ROC curve analysis of exosomal ZIP4 between BP and N groups. N, ROC curve analysis of exosomal ZIP4 between BP and B groups. *$P < .05$; **$P < .01$; ***$P < .001$; $P < .0001$

analyze the differentially expressed exosomal proteins (Figure 2C,D). The results showed that PC-1.0-derived exosomal proteins are more enriched in proteasome, focal adhesion and extracellular matrix regulation.

3.3 | Exosomal ZIP4 is a potential regulator and novel biomarker for pancreatic cancer

ZIP4 and several other proteins from the proteomic analysis were chosen for further validation with exosomes from PC-1.0/PC-1 cell lines and human pancreatic cancer cell lines with similar biological characteristics (AsPC-1 and Capan-2). Western blot analysis of these selected exosomal proteins showed consistent results indicating that our proteomic analysis was reliable (Figure 3A). Representative electron microscope images and exosomal biomarker validation of the exosomes from AsPC-1 and Capan-2 cells are presented in Figure 3B,C. Multiple bioinformatics tool sites were used to analyze the clinical data on ZIP4 from The Cancer Genome Atlas (TCGA) database. Analysis by GEPIA showed that SLC39A4 (a gene encoding ZIP4 in humans) was highly expressed in multiple cancers, including pancreatic cancer (Figure 3D). Analysis by UALCAN showed that ZIP4 was differentially expressed in cases with different stages of pancreatic cancer, pancreatitis status and healthy subjects (Figure 3E-G).[40] Cases with higher expression levels of ZIP4 showed worse survival rates than cases with lower expression levels (Figure 3H). Analysis by cBioPortal showed that cases with alterations in SLC39A4 were associated with less progression/disease-free time than those without, indicating that ZIP4 was closely correlated with pancreatic cancer progression (Figure 3I).[41,42]

3.4 | Exosomal ZIP4 promotes pancreatic cancer cell growth in vitro

We cocultured PC-1 cells with different amounts (equaling 20, 40 and 60 μg of exosomal proteins) of PC-1.0-derived exosomes. The expression level of ZIP4 in PC-1 cells rose with increased amounts of added exosomes (Figure 4A). The results indicated that PC-1.0 cells could deliver ZIP4 into PC-1 cells through the exosomes. Next, we investigated the role of exosomal ZIP4 in pancreatic cancer progression by comparing the functions of normal and ZIP4 knockdown exosomes from PC-1.0 cells in vitro (Figure 4B). The CCK-8 cell proliferation assay, wound-healing migration assay, and

Transwell invasion assay were performed to investigate the potential functions of exosomal ZIP4. The results showed that exosomal ZIP4 significantly enhanced PC-1 cell proliferation and migration abilities but not invasion abilities (Figure 4C-E). Further cell cycle analysis showed that DNA synthesis was enhanced in the PC-1 cells by exosomal ZIP4 (Figure 4F). These results indicated that exosomal ZIP4 may promote pancreatic cancer progression mainly through enhancing growth.

3.5 | Exosomal ZIP4 promotes pancreatic cancer growth in a nude mice model

Based on our in vitro results, we further investigated whether exosomal ZIP4 could promote cancer growth in an in vivo environment. The PC-1 cells were subcutaneously injected into the right axilla area of nude mice according to the experimental design shown in Figure 5A. The nude mice that were further injected with normal PC-1.0-derived exosomes showed more rapid and larger tumor growth in the subcutaneous implantation sites than those injected with ZIP4-knockdown exosomes (Figure 5B-D). Furthermore, both western blot and immunohistochemical assays showed that the nude mice injected with normal PC-1.0-derived exosomes expressed higher levels of ZIP4 in the tumor tissue than those injected with ZIP4-knockdown exosomes, indicating a correlation between ZIP4 expression and tumor growth (Figure 5E,F).

3.6 | Exosomal ZIP4 is a novel diagnostic biomarker for pancreatic cancer

Finally, we investigated the efficacy of exosomal ZIP4 as a novel diagnostic biomarker for pancreatic cancer with serum samples from patients with malignant pancreatic cancer (MP, n = 24), patients with benign pancreatic disease (BP, n = 32, 18 benign pancreatic tumor-BPC, 14 pancreatitis-P), patients with biliary disease (B, n = 32), and normal controls (N, n = 46). Patients with biliary disease were also included, as they showed early symptoms similar to those of pancreatic diseases, such as jaundice, anorexia, nausea and elevated CA19-9 levels. The general characteristics of our participants are summarized and depicted with bar charts (Figure 6A-D), while their clinical information is provided in an Excel chart (Supplementary Table S2). Representative electron microscope images and exosomal biomarker validation of serum-derived exosomes are provided (Figure 6E,F). ELISA showed that the level of exosomal ZIP4 was significantly higher in the serum from the MP group than in the serum from the BP ($P < .0001$), B ($P = .0053$) and N ($P < .0001$) groups. No significant difference was found between the BP and N ($P = .5940$), BP and B ($P = .2536$), or B and N ($P = .1068$) groups (Figure 6G). We further found a significant difference between the exosomal ZIP4 levels in the BPC and P groups ($P = .0356$) (Figure 6H). Receiver operating characteristic (ROC) curve analysis was further performed to evaluate the efficacy of exosomal ZIP4 as a diagnostic biomarker for pancreatic cancer. The areas under the curve (AUC) showed promising diagnostic efficacy between the MP and N groups (AUC = .8931) and the MP and BP groups (AUC = .89) and showed acceptable diagnostic efficacy between the MP and B groups (AUC = .8112) (Figure 6I-K). The diagnostic efficacy of exosomal ZIP4 was low between the BPC and P (AUC = .6944), BP and N (AUC = .5965), and BP and B (AUC = .5703) groups (Figure 6L-N).

4 | DISCUSSION

In this study, we found that PC-1.0-derived exosomes could significantly enhance the proliferation, migration and invasion abilities of PC-1 cells. These results indicated that highly malignant pancreatic cancer cells may transport their oncogenic characteristics to less malignant cancer cells through the exosomes to accelerate disease progression. Blocking exosome-mediated transportation may slow cancer progression and elevate the overall survival rate. Next, we compared the exosomal protein profiles of these 2 cell lines and identified ZIP4 as the most upregulated exosomal proteins in PC-1.0 cells. Compared with using 2 random cancer cell lines with different degrees of malignancy, our isogenic cell lines possessed unique superiority. The differential exosomal proteins between PC-1.0 and PC-1 cells were correlated more with cancer progression than with individual differences. Finally, we investigated the efficacy of exosomal ZIP4 as a novel diagnostic biomarker for pancreatic cancer. The limited sample volume of the current study led to a potentially unrepresentative conclusion, as only 1 of the 18 patients with benign pancreatic tumors was male. The number of female patients with pancreatic cancer was double that of male patients. The current study requires further validation with a larger sample volume collected by multiple centers and elucidation of the underlying signaling pathway of exosomal ZIP4.

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CONFLICT OF INTEREST

The authors declare no conflict of interest for this article.

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REFERENCES

1. Babic A, Wolpin BM. Circulating exosomes in pancreatic cancer: will they succeed on the long, littered road to early detection marker? *Clin Chem*. 2016;62:307-309.
2. Shaib Y, Davila J, Naumann C, El-Serag H. The impact of curative intent surgery on the survival of pancreatic cancer patients: a U.S. Population-based study. *Am J Gastroenterol*. 2007;102:1377-1382.

3. Hidalgo M. Pancreatic cancer. *N Engl J Med*. 2010;362:1605-1617.
4. Tan X, Liu P, Huang Y, et al. Phosphoproteome analysis of invasion and metastasis-related factors in pancreatic cancer cells. *PLoS ONE*. 2016;11:e0152280.
5. Tan X, Egami H, Ishikawa S, et al. Arrangement of expression and distribution of tight junction protein claudin-1 in cell dissociation of pancreatic cancer cells. *Int J Oncol*. 2004;25:1567-1574.
6. Tan X, Egami H, Ishikawa S, et al. Involvement of matrix metalloproteinase-7 in invasion-metastasis through induction of cell dissociation in pancreatic cancer. *Int J Oncol*. 2005;26:1283-1289.
7. Tan X, Egami H, Kamohara H, et al. Involvement of the mitogen-activated protein kinase kinase 2 in the induction of cell dissociation in pancreatic cancer. *Int J Oncol*. 2004;24:65-73.
8. Tan X, Zhou L, Wang W, et al. Genomic analysis of invasion-metastasis-related factors in pancreatic cancer cells. *Exp Ther Med*. 2010;1:211-216.
9. Hirota M, Egami H, Corra S, et al. Production of scatter factor-like activity by a nitrosamine-induced pancreatic cancer cell line. *Carcinogenesis*. 1993;14:259-264.
10. Liu P, Weng Y, Sui Z, et al. Quantitative secretomic analysis of pancreatic cancer cells in serum-containing conditioned medium. *Sci Rep*. 2016;6:37606.
11. Thery C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. *Nat Rev Immunol*. 2002;2:569-579.
12. Jin H, Wu Y, Tan X. The role of pancreatic cancer-derived exosomes in cancer progress and their potential application as biomarkers. *Clin Transl Oncol*. 2017;19:921-930.
13. Tkach M, Thery C. Communication by extracellular vesicles: where we are and where we need to go. *Cell*. 2016;164:1226-1232.
14. Costa-Silva B, Aiello NM, Ocean AJ, et al. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. *Nat Cell Biol*. 2015;17:816-826.
15. Fong MY, Zhou W, Liu L, et al. Breast-cancer-secreted miR-122 reprograms glucose metabolism in premetastatic niche to promote metastasis. *Nat Cell Biol*. 2015;17:183-194.
16. Zhang L, Zhang S, Yao J, et al. Microenvironment-induced PTEN loss by exosomal microRNA primes brain metastasis outgrowth. *Nature*. 2015;527:100-104.
17. Liu Y, Cao X. Characteristics and significance of the pre-metastatic niche. *Cancer Cell*. 2016;30:668-681.
18. Zomer A, Maynard C, Verweij FJ, et al. In vivo imaging reveals extracellular vesicle-mediated phenocopying of metastatic behavior. *Cell*. 2015;161:1046-1057.
19. Becker A, Thakur BK, Weiss JM, Kim HS, Peinado H, Lyden D. Extracellular vesicles in cancer: cell-to-cell mediators of metastasis. *Cancer Cell*. 2016;30:836-848.
20. Hoshino A, Costa-Silva B, Shen TL, et al. Tumour exosome integrins determine organotropic metastasis. *Nature*. 2015;527:329-335.
21. Peinado H, Aleckovic M, Lavotshkin S, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat Med*. 2012;18:883-891.
22. Théry C. Diagnosis by extracellular vesicles. *Nature*. 2015;523:161-162.
23. Melo SA, Luecke LB, Kahlert C, et al. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature*. 2015;523:177-182.
24. Thakur BK, Zhang H, Becker A, et al. Double-stranded DNA in exosomes: a novel biomarker in cancer detection. *Cell Res*. 2014;24:766-769.
25. Zhang Y, Chen C, Yao Q, Li M. ZIP4 upregulates the expression of neuropilin-1, vascular endothelial growth factor, and matrix metalloproteases in pancreatic cancer cell lines and xenografts. *Cancer Biol Ther*. 2010;9:236-242.
26. Li M, Zhang Y, Bharadwaj U, et al. Down-regulation of ZIP4 by RNA interference inhibits pancreatic cancer growth and increases the survival of nude mice with pancreatic cancer xenografts. *Clin Cancer Res*. 2009;15:5993-6001.
27. Zhang Y, Bharadwaj U, Logsdon CD, Chen C, Yao Q, Li M. ZIP4 regulates pancreatic cancer cell growth by activating IL-6/STAT3 pathway through zinc finger transcription factor CREB. *Clin Cancer Res*. 2010;16:1423-1430.
28. Yang J, Zhang Y, Cui X, et al. Gene profile identifies zinc transporters differentially expressed in normal human organs and human pancreatic cancer. *Curr Mol Med*. 2013;13:401-409.
29. Zhang Y, Yang J, Cui X, et al. A novel epigenetic CREB-miR-373 axis mediates ZIP4-induced pancreatic cancer growth. *EMBO Mol Med*. 2013;5:1322-1334.
30. Xu X, Guo HJ, Xie HY, et al. ZIP4, a novel determinant of tumor invasion in hepatocellular carcinoma, contributes to tumor recurrence after liver transplantation. *Int J Biol Sci*. 2014;10:245-256.
31. Lin Y, Chen Y, Wang Y, et al. ZIP4 is a novel molecular marker for glioma. *Neuro Oncol*. 2013;15:1008-1016.
32. Huang C, Cui X, Sun X, Yang J, Li M. Zinc transporters are differentially expressed in human non-small cell lung cancer. *Oncotarget*. 2016;7:66935-66943.
33. Weaver BP, Zhang Y, Hiscox S, et al. Zip4 (Slc39a4) expression is activated in hepatocellular carcinomas and functions to repress apoptosis, enhance cell cycle and increase migration. *PLoS ONE*. 2010;5:e13158.
34. Egami H, Takiyama Y, Cano M, Houser WH, Pour PM. Establishment of hamster pancreatic ductal carcinoma cell line (PC-1) producing blood group-related antigens. *Carcinogenesis*. 1989;10:861-869.
35. Hood JL, Pan H, Lanza GM, Wickline SA. Consortium for translational research in Advanced I, nanomedicine. Paracrine induction of endothelium by tumor exosomes. *Lab Invest*. 2009;89:1317-1328.
36. You YW, Shan Y, Chen J, et al. Matrix metalloproteinase 13-containing exosomes promote nasopharyngeal carcinoma metastasis. *Cancer Sci*. 2015;106:1669-1677.
37. Ashburner M, Ball CA, Blake JA, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet*. 2000;25:25-29.
38. The Gene Ontology Consortium. Expansion of the gene ontology knowledgebase and resources. *Nucleic Acids Res*. 2017;45:D331-D338.
39. Koonin EV, Fedorova ND, Jackson JD, et al. A comprehensive evolutionary classification of proteins encoded in complete eukaryotic genomes. *Genome Biol*. 2004;5:R7.
40. Chandrashekar DS, Bashel B, Balasubramanya SAH, et al. UALCAN: a portal for facilitating tumor subgroup gene expression and survival analyses. *Neoplasia*. 2017;19:649-658.
41. Gao J, Aksoy BA, Dogrusoz U, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal*. 2013;6:pl1.
42. Cerami E, Gao J, Dogrusoz U, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov*. 2012;2:401-404.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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