CircFOXK2 Promotes Growth and Metastasis of Pancreatic Ductal Adenocarcinoma by Complexing with RNA-Binding Proteins and Sponging MiR-942

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ABSTRACT

The detailed biological functions of circular RNA (circRNA) are largely unexplored. Using circRNA sequencing, we identified 169 differentially expressed circRNA in pancreatic ductal adenocarcinoma (PDAC) cells compared with nontumor human pancreatic ductal epithelial cells. Among them, circFOXK2 was validated with significant upregulation in PDAC cells and 63% of primary tumors (53 of 84). circFOXK2 promoted cell growth, migration, and invasion and was involved in cell-cycle progression and apoptosis. circFOXK2 contained multiple miRNA binding sites, functioning as a sponge for miR-942, which in turn promoted expression of ANK1, GDNF, and PAX6. A novel and highly specific circRNA-pulldown followed by mass spectrometry analysis identified 94 circFOXK2-interacting proteins, which were involved in cell adhesion, mRNA splicing, and structural molecule activity. Of these, circFOXK2 interactions with YBX1 and hnRNPK enhanced expression of oncogenes NUF2 and PDXX. Knockdown of circFOXK2 reduced binding of YBX1 and hnRNPK to NUF2 and PDXX, in turn decreasing their expression. Collectively, our findings demonstrate that circFOXK2 in complex with YBX1 and hnRNPK promotes expression of oncogenic proteins that contribute to PDAC progression.

Significance: This study reveals a prominent role for the circRNA circFOXK2 in PDAC progression, suggesting that circFOXK2 might be a novel diagnostic marker for PDAC.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the United States and Europe (1, 2). Surgery, chemotherapy, and radiotherapy are standard therapy for PDAC. Unfortunately, patients with PDAC can only extend survival up to several months (3, 4). Also, combining gemcitabine with other chemotherapy drugs such as nab-paclitaxel can only provide a limited extension of survival with developing adverse effects including leukopenia and peripheral neuropathy (5). Therefore, novel therapeutic targets are urgently needed for treating PDAC.

Circular RNA (circRNA), which was first discovered in 1976, is a type of noncoding RNA with unknown function described previously. circRNA is formed by the linking of 3' end of exon back to its 5' end, forming a circular structure. cirR-7 was identified as the first functional circRNA, which acted as a miRNA sponge to inhibit miR-7 activity in brain (6, 7). Since then, evidence has been obtained regarding expression patterns and functional roles of circRNAs in various biological processes including cancer progression. F-circSR1 and F-circSR2 promoted cell migration in lung cancer (8). CircIRAK3 facilitated breast cancer metastasis through sponging miR-3607 (9). circJ-catenin promoted cell proliferation by activating Wnt pathway in liver cancer (10). However, the importance of circRNAs in PDAC development is not fully understood.

In this study, we identified 169 differentially expressed circRNAs in PDAC cells by circRNA sequencing. We validated that one of the circRNAs, circFOXK2, was significantly upregulated in both PDAC cells and primary tumors. circFOXK2 promoted cell growth, clonogenic ability, migration, invasion, and liver metastasis in PDAC. Also, circFOXK2 functioned as a miRNA sponge for miR-942, and in turn promoted expression of ankyrin 1 (ANK1), Glial cell-derived neurotrophic factor (GDNF), and paired box 6 (PAX6). Importantly, circRNA-pulldown and mass spectrometry identified 94 circFOXK2-interacting proteins to multiple biological processes including cell adhesion, mRNA splicing, and structural molecule activity. We demonstrated that circFOXK2 complexed with Y-box binding protein 1 (YBX1) and heterogeneous nuclear ribonucleoprotein K (hnRNPK) to promote the expression of oncogenic proteins NUF2 component of NDC80 kinetochore complex (NUF2) and pyridoxal kinase (PDXX) in PDAC.

Materials and Methods

Mammalian cell lines and clinical samples
PDAC cell lines PANC1, SW1990, CAPAN-2, CFPAC1, and BxPC-3, and HEK293 cells were obtained from ATCC. The human pancreatic ductal epithelial (HPDE) cell line was a gift from Dr. Ming-Sound Tsao (University Health Network, Ontario Cancer Institute and Princess Margaret Hospital Site, Toronto, Canada; ref. 11). All cell lines were verified by short tandem repeat profiling at the GENEWIZ, Inc. within 6 months of use and were cultured under the condition as described previously (12). All cell lines undergo routine Mycoplasma testing. 84 pairs of PDAC primary tumor and adjacent nontumor
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cancers are obtained from patients who underwent pancreatic resection at the Prince of Wales Hospital, Hong Kong. The study was carried out according to the ethical guidelines and with the approval of the Joint CUHK-NTEC Clinical Research Ethics Committee in accordance with Declaration of Helsinki and the written informed consent was obtained from all patients recruited. All specimens were fixed and embedded into paraffin.

**circRNA sequencing and identification**

circRNA sequencing was performed to analyze the expression pattern of circRNAs in HPDE, PANC1, and SW1990 cells. The total RNA extracted from cells was depleted from ribosomal RNA to linear RNA by RNeasy R. Then RNA was fragmented and was reverse transcribed. After linking with the sequencing adaptor and PCR amplification, a library was constructed for the circRNA sequencing. The raw sequencing reads from each sample were first mapped to the reference human genome using TopHat2 (13). The unmapped reads were extracted and mapped to reference human genome using TopHat-Fusion (14). Reads were processed into two anchors from both ends of the reads. Anchors that were aligned into the same chromosome but in reversed orientation were considered as the potential back-spliced junction reads. Because the common back-spliced junctions were GT/AG, GC/AG, and AT/AC, back-spliced junction reads with these junctions were extracted. Mapped reads (from TopHat2) and back-spliced reads (from TopHat-Fusion) were used to quantify the abundance of each circRNA candidate, denoted in RPM (reads per million mapped reads). DESeq2 was used to compare the expression level of each circRNA between samples. circRNA sequencing data are available in the NCBI Gene Expression Omnibus under accession number GSE135731.

**Plasmid and oligonucleotide transfection**
circRNA overexpression plasmid was constructed by cloning the FOXK2 exon 2 and 3 into pcDNA3.1 (+) circRNA mini vector, which was a gift from Jeremy Wilusz (Addgene plasmid, catalog no. 60648; ref. 15). pmiR-Reporter plasmid with the inclusion of circRNA sequence in the 3’ untranslated region for luciferase assay was constructed by cloning circRNA sequence into region directly downstream of the firefly luciferase gene in the pmiR-Reporter (Promega). Mutation of the miRNA binding site in the pmiR-Reporter plasmid was generated using KAPA HiFi DNA Polymerase (Kapa Biosystem) and primers with mutation sites. The lentiviral vector for stable knockdown of circFOXK2 was generated by cloning the short hairpin RNA (shRNA) sequence targeting circFOXK2 into the lentiviral vector with H1 promoter as described previously (16). miRNAs and siRNAs were purchased from GenePharma. Plasmids, miRNA mimics, and siRNA transfections were performed by lipofectamine 3000 (Invitrogen). For comparison, Firefly luciferase activity was first normalized with Renilla luciferase activity. Then, the effect of miRNAs on pmiR-Reporter with circRNA was normalized with pmiR-Reporter without circRNA. Finally, fold change was calculated by comparing the effect of miRNA mimics with miRNC.

**miRNA pulldown**
miRNA pulldown assay was performed by transfecting PANC1 cells with 100 nmol/L 3’-end biotinylated miRNA mimics. After 24 hours, the cells were washed twice with iced PBS, followed by cell lysis using miRNA pulldown lysis buffer (20 mmol/L Tris-HCl at pH 7.5, 100 mmol/L KCl, 5 nmol/L MgCl2, 0.3% NP-40). In the miRNA pulldown assay, 25 μL Dynabeads MyOne Streptavidin C1 (Thermo Fisher Scientific) was washed three times with miRNA pulldown lysis buffer. Then the beads were blocked with 1 mg/mL yeast tRNA (Thermo Fisher Scientific) and 1 mg/mL BSA at 4°C for 2 hours with rotation. After that, the beads were washed twice with miRNA pulldown lysis buffer. Biotin-labeled miRNAs were isolated by incubating the beads with 100 μL cell lysate and 100 μL miRNA pulldown lysis buffer at 4°C for 4 hours with rotation. The beads were washed twice with miRNA pulldown lysis buffer. Biotin-labeled miRNAs and their interacting RNAs were isolated by TRIzol Reagent. Detection of miRNA-interacting RNAs was performed by qRT-PCR.

**In vitro transcription**
circRNA overexpression plasmid was digested with XhoI (NEB) to form a linearized template DNA. Then in vitro transcription of

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**In vivo subcutaneous injection**
Male BALB/c nude mice ages 4 to 6 weeks were acquired from Laboratory Animal Services Centre of the Chinese University of Hong Kong (Shatin, Hong Kong). Animal handling and experimental procedures were approved by the Animal Experimental Ethics Committee of the institute. For tumor growth assay, 6 × 10^5 cells were resuspended in 1 × PBS with 20% Matrigel (Corning) and were injected subcutaneously into the right flank of the nude mice (7 mice/group). After tumor formation, tumor growth was monitored every 3–4 days, and the tumor volume was measured by a caliper and calculated by the equation: volume = (length × width^2)/2.

**In vivo orthotopic injection**
Male BALB/c nude mice ages 4 to 6 weeks were acquired from Laboratory Animal Services Centre of the Chinese University of Hong Kong (Shatin, Hong Kong). Animal handling and experimental procedures were approved by the Animal Experimental Ethics Committee of the institute. For tumor metastasis assay, 5 × 10^5 cells were resuspended in 1 × PBS with 20% Matrigel and were injected orthotopically to the head of the pancreas (17). Tumors and organs were collected and examined for metastasis.

**Luciferase assay**
HEK293 cells were seeded in 96-well plate. After 24 hours, cells were cotransfected with 100 ng pmiR-Reporter plasmid, 1 ng Renilla luciferase reporter plasmid, and miRNA mimics. Each miRNA mimics or miRNC was cotransfected with pmiR-Reporter with or without circRNA sequence. After 72 hours, cells were washed twice with iced PBS and luciferase activity was measured with Nano-Glo Dual-Luciferase Reporter Assay System (Promega). For comparison, Firefly luciferase activity was first normalized with Renilla luciferase activity. Then, the effect of miRNAs on pmiR-Reporter with circRNA was normalized with pmiR-Reporter without circRNA. Finally, fold change was calculated by comparing the effect of miRNA mimics with miRNC.

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**qRT-PCR**
The cytoplasmic and nuclear fractions were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). RNA from whole-cell lysate or cell fractions was isolated by TRIzol Reagent. Formalin-fixed, paraffin-embedded (FFPE) sample RNA was isolated by miRNeasy FFPE Kit (Qiagen) according to the manufacturer’s protocol. Measurement of gene expression level was performed by qRT-PCR. Reverse transcription of total RNA (except miRNA) was performed by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Reverse transcription of miRNA was performed by Mir-X miRNA First-Strand Synthesis Kit (Takara). qRT-PCR was performed by ABI 7900HT Real-Time PCR system using SYBR Green PCR Master Mix (Applied Biosystems). The primers used in this study were listed in Supplementary Table S2.

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**Declaration of Helsinki and the written informed consent** was obtained from all patients recruited. All specimens were fixed and embedded into paraffin.

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**In vitro transcription**
circRNA overexpression plasmid was digested with XhoI (NEB) to form a linearized template DNA. Then in vitro transcription of
circRNAs and their parental mRNAs was performed by MEGA script T7 Transcription Kit (Thermo Fisher Scientific; ref. 16). 1 μg template DNA was mixed with 1 μl of ATP solution, 1 μl of CTP solution, 1 μl of GTP solution, 0.9 μl of UTP solution, 0.15 μl of biotin-UTP solution (Epicentre), 1 μl of enzyme mixture, and RNase-free water to 10 μl. After in vitro transcription at 37°C overnight, template DNA was digested with DNase for 15 minutes at 37°C. For circRNA transcription, parental mRNA was digested by 10 U RNase R (Epicentre) for 5 hours at 37°C. Digestion was terminated by ammonium acetate stop solution and in vitro transcribed RNAs were purified by phenol:chloroform:isoamylalcohol solution according to the manufacturer's protocol.

RNA pulldown
RNA pulldown assay for investigating RNA-protein interaction was performed by Pierce Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific). 5 μg of the in vitro transcribed RNA was heated at 90°C for 2 minutes in 1× RNA capture buffer, followed by incubation on ice for 2 minutes and at room temperature for 30 minutes. Then the biotin-labeled RNA was mixed with 75-μl streptavidin magnetic beads, which were prewashed twice with Tris solution (Epicentre), 1 μl of RNase R (Epicentre) was digested by RNase R, which specifically degrades linear RNAs but not circRNAs (ref. 16). 1 μl of biotin-UTP solution (Epicentre), 1 μl of enzyme mixture, and RNase-free water to 10 μl. After in vitro transcription at 37°C overnight, template DNA was digested with DNase for 15 minutes at 37°C. For circRNA transcription, parental mRNA was digested by 10 U RNase R (Epicentre) for 5 hours at 37°C. Digestion was terminated by ammonium acetate stop solution and in vitro transcribed RNAs were purified by phenol:chloroform:isoamylalcohol solution according to the manufacturer's protocol.

RNA immunoprecipitation
RNA immunoprecipitation was performed by Magna RIP RNA Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer's protocol. Briefly, cells were washed twice with ice-cold PBS, followed by cell lysis using equal volume of RIP lysis buffer. Magnetic beads were washed twice with RIP wash buffer, followed by incubation with 2 μg antibody against hnRNPK (rabbit; Proteintech 11426-1-AP), YBX1 (rabbit; Proteintech 20339-1-AP), SEPT11 (rabbit; Proteintech 14672-1-AP), ILF3 (rabbit; Proteintech 19867-1-AP), ASF (rabbit; Proteintech 12929-2-AP), and RAB11FIP1 (rabbit; Proteintech 16778-1-AP) for 30 minutes at room temperature. Immunoprecipitation was performed by incubating cell lysate with the magnetic bead–antibody complex overnight at 4°C. Then the beads were washed six times with RIP wash buffer, followed by proteinase K digestion at 37°C for 30 minutes. RNA was purified by phenol:chloroform:isoamylalcohol (15593-031, Invitrogen). qRT-PCR was used to analyze the enrichment of RNAs with target proteins.

Statistical analysis
Statistical analysis was performed by GraphPad Prism 7. Student t test, χ² t test and Pearson correlation were used as appropriate. Data were shown in mean ± SD. Statistically significant was considered when P value (two-sided) was less than 0.05.

Data Availability
Supplementary methods are described in Supplementary information.

Results
Identification of circRNAs in PDAC cells
To identify the differentially expressed circRNAs in PDAC, we performed circRNA sequencing of nontumor HPDE and PDAC cells PANC1 and SW1990 (accession number GSE135731). In total, 17,158 circRNAs were identified in which 84% were exonic (Supplementary Fig. S1A). Among them, 83 upregulated and 86 downregulated circRNAs were revealed in PANC1 and SW1990 cells in comparison with nontumor HPDE cells (Supplementary Fig. S1B). To validate the results from circRNA sequencing and the clinical significances of the upregulated circRNAs, we measured the expressions of selected circRNAs in a panel of PDAC cells and primary tumors (Supplementary Fig. S1C and S1D). circFOXK2 was found with significant upregulation in PDAC cells (Fig. 1A), as well as 63% of PDAC primary tumors (53 of 84; Fig. 1B).

Characterization of circFOXK2
Next, we examined the physical circular structure of circFOXK2. circFOXK2 is formed by the back-splicing of exon 2 and 3 of Forkhead box protein K2 (FOXK2; Fig. 1C). Outward-facing divergent primer and inward-facing convergent primer were designed to validate the formation of circFOXK2. Both primers amplified a product of expected size from cDNA, while only convergent primers resulted in an amplification from genomic DNA (gDNA; Fig. 1D). The presence of back-splicing junction was confirmed by Sanger sequencing (Fig. 1E). Also, circFOXK2 was observed to resist the digestion by RNase R, which specifically degraded linear RNAs but not circRNAs (Fig. 1F). Reduction in reverse-transcription efficiency by oligo-dT primers due to the lack of polyA tail also demonstrated the circularity of circFOXK2 (Fig. 1G). These results suggested the formation of circFOXK2 was not due to genomic rearrangement. Owing to its circular structure, we found that circFOXK2 was more stable than FOXK2 (Fig. 1H). Localization of circFOXK2 was examined by measuring its level in cytoplasm and nucleus, which demonstrated the enrichment of circFOXK2 in the cytoplasm of PDAC cells (Fig. 1I). Coding potential analysis suggested the lack of protein coding ability of circFOXK2 (Supplementary Table S3). Our results validated the circularity of circFOXK2.
circFOXK2 promotes cell growth and invasion in vitro

To elucidate the functions of circFOXK2 in PDAC, we employed siRNA, which specifically targeted the back-splicing junction of circFOXK2, without altering the expression of its parental gene (Supplementary Fig. S2A). Knockdown of circFOXK2 inhibited cell growth and clonogenic ability of PDAC cells (Fig. 2A–C). The repressed cell growth by knockdown of circFOXK2 was due to cell-cycle arrest in G0–G1 phase and induction of apoptosis (Fig. 2D). Annexin V staining, cleavage of PARP and caspase-3, and decrease in Bcl2 level also demonstrated the induction of apoptosis after knockdown of circFOXK2 in PDAC cells (Fig. 2E and F). Also, knockdown of circFOXK2 inhibited cell migration and invasion (Fig. 2G and H). Furthermore, we constructed stable knockdown CFPAC1 cells using sh-circFOXK2 lentiviral system. Stable knockdown of circFOXK2 inhibited cell growth, clonogenic ability, and invasiveness (Supplementary Fig. S2B–S2E). On the other hand, overexpression of circFOXK2 in HPDE cells significantly promoted cell growth, migration, and invasion. (Supplementary Fig. S3A–S3E). Collectively, our results demonstrated the importance of circFOXK2 in promoting PDAC cell growth and invasion.

circFOXK2 promotes tumor growth and metastasis in vivo

Knockdown of circFOXK2 was also employed for in vivo study. Xenograft mouse model was generated by subcutaneous injection of sh-circFOXK2 CFPAC1 cells. Knockdown of circFOXK2 by shRNA significantly inhibited tumor growth (Fig. 3A–E). Because results
from in vitro experiments revealed that circFOXK2 promoted PDAC cell migration and invasion, we constructed PDAC metastatic mice model through orthotopic injection of sh-circFOXK2 CFPAC1 cells to the pancreas. Knockdown of circFOXK2 inhibited the PDAC metastasis to liver (Fig. 3F). Our results found that that circFOXK2 played critical roles in promoting PDAC growth and liver metastasis.
Identification of circFOXK2-interacting miRNAs

We next investigated the detailed mechanism of circFOXK2 in PDAC. Because many cytoplasmic circRNAs serve as “microRNA sponges” in regulating cancer progression, including liver, lung, and breast cancer (8–10), we examined whether circFOXK2 could function as a miRNA sponge. First, we performed the bioinformatic analysis by TargetScan (18) to reveal potential miRNA binding sites on circFOXK2. To validate the circFOXK2–miRNA interaction, luciferase reporter assay using circFOXK2-luciferase reporter and miRNA mimics was performed (Fig. 4A), which demonstrated a reduction in luciferase activity with miR-942 mimics (Fig. 4B). Mutating the miR-942 binding sites restored the luciferase activity (Fig. 4C). circFOXK2–miRNA interaction was further validated by miRNA pull-down. Biotin-labeled miR-942 mimics could significantly enrich circFOXK2 in PANC1 cells (Fig. 4D). These results suggested the interaction between circFOXK2 and miR-942 in PDAC cells.

To study the functions of circFOXK2–miR-942 interaction in PDAC, we first knocked down circFOXK2, which leads to a subsequent increase in miR-942 expression level (Fig. 4E). Conversely, transfecting miR-942 mimics did not alter the expression of circFOXK2, but downregulated the targets of miR-942: ANK1, GDNF, and PAX6 (Fig. 4F; Supplementary Fig. S4A and S4B). These results suggested that circFOXK2 inhibited the activity of miR-942 in PDAC. As the role of miR-942 in PDAC is unclear, we attempted to study the importance of miR-942 in PDAC. We found that miR-942 was frequently downregulated in PDAC cells and primary tumors (Supplementary Fig. S5A–S5C). Also, we found that transfecting miR-942 mimics inhibited cell growth and clonogenic ability in PDAC cells (Supplementary Fig. S5D and S5E). Our results illustrated the role of miR-942 in promoting PDAC cell growth.

Because circFOXK2 functioned as a miRNA sponge for miR-942, the expression level of ANK1, GDNF, and PAX6 were measured after knockdown or overexpression of circFOXK2. Knockdown of circFOXK2 decreased the expression levels of ANK1, GDNF, and PAX6 in PDAC cells, while overexpression of circFOXK2 promoted the expression of ANK1, GDNF, and PAX6 in HPDE cells (Fig. 5A and B; Supplementary Fig. S6A–S6D). The effect of circFOXK2 overexpression on GDNF and PAX6 could be rescued by the introduction of miR-942 mimics (Fig. 5C). Also, in the mice tumors with circFOXK2 knocked down, ANK1, GDNF, and PAX6 were downregulated (Fig. 5D; Supplementary Fig. S6E). Analysis of PDAC primary tumors also revealed the positive correlation between circFOXK2, ANK1, and
Collectively, our results suggested that circFOXK2 inhibited miR-942, and in turn promoted the expression of ANK1, GDNF, and PAX6 in PDAC.

**Identification of circFOXK2-interacting proteins**

Many studies have demonstrated the major role of circRNA through functioning as miRNA sponges, whereas the binding capability of circRNA on protein in regulating gene expression remains uncertain. Therefore, we next studied the importance of circRNA–protein interaction in PDAC. We in vitro transcribed circFOXK2, which functioned as a probe to pull-down circFOXK2-interacting proteins. To eliminate the “false positive” of the pull-down proteins, we constructed additional two negative controls: (i) RNA probe with an identical sequence as circFOXK2, without the formation of circRNAs; (ii) RNA probe with similar secondary structure as circRNA, which was circGFP (Fig. 6A). Therefore, the proteins pulled down by circFOXK2 were due to the unique sequence and secondary structure of circFOXK2.

Mass spectrometry analysis identified 94 circFOXK2-interacting proteins in PANC1 cells (accession number PXD015048; Fig. 6B). Also, protein–protein interactions (PPI) of the circRNA-interacting proteins were analyzed in the STRING database (19). 101 PPIs were observed for circFOXK2-interacting proteins (Supplementary Fig. S7A). Gene ontology analysis also revealed that the circFOXK2-interacting proteins play important roles in many biological processes, including cell adhesion, mRNA splicing, and structural molecule activity (Supplementary Fig. S7B). Our results suggested that circFOXK2 interacted with multiple proteins with critical roles in PDAC.

**circFOXK2 promotes the expression of oncogenic proteins via complexing YBX1 and hnRNPK**

RNA immunoprecipitation assay was performed in PDAC cells to validate the results from mass spectrometry analysis. circFOXK2 was significantly enriched by YBX1 and hnRNPK (Fig. 6C). Because YBX1 complexed with hnRNPK in regulating gene expression to promote PDAC progression (20; Supplementary Fig. S8), we hypothesized that circFOXK2 interacted with YBX1 and hnRNPK complex to promote PDAC development. Publicly available datasets of knockdown of YBX1 were first employed to identify genes that were consistently regulated by YBX1 in multiple cancers (Supplementary Fig. S9A). RNA immunoprecipitation assay with YBX1 and hnRNPK demonstrated the enrichment of Coronin 1C (CORO1C), NUF2, PDXK, and Protein Phosphatase 2 Regulatory Subunit B gamma (PPP2R2C), indicating they were the direct targets of YBX1 and hnRNPK complex.
Supplementary Fig. S9B). Consistently, knockdown of YBX1 significantly downregulated these targets in CFPAC1 cells (Supplementary Fig. S9C). Analysis of The Cancer Genome Atlas dataset also revealed the upregulation of PPP2R2C, PDXK, and NUF2, and their positive correlations to YBX1 expression in PDAC (Supplementary Fig. S9D–S9F). Importantly, NUF2 and PDXK were downregulated after the knockdown of circFOXK2 while they were significantly enriched by circFOXK2 pulldown (Fig. 6D and E). The effect of circFOXK2 overexpression on NUF2 and PDXK expressions could be rescued by the knockdown of YBX1 and hnRNPK (Fig. 6F). Knockdown of circFOXK2 decreased the interaction of YBX1 and hnRNPK to NUF2 and PDXK (Fig. 6G and H). Analysis of PDAC cells and primary tumors revealed the upregulation of PDXK and NUF2, and the positive correlation between NUF2 and circFOXK2 expression (Fig. 6I; Supplementary Fig. S10A and S10B). Also, knockdown of NUF and PDXK significantly inhibited PDAC cell growth and invasion (Supplementary Fig. S10C–S10E). Collectively, our results suggested that circFOXK2, YBX1, and hnRNPK complex interacted and promoted the expressions of oncogenic proteins NUF2 and PDXK in PDAC.

Discussion

In this study, we profiled circRNAs expressions in nontumor HPDE and PDAC cells by circRNA sequencing. We identified 169 differentially expressed circRNAs in PDAC. Further validation experiments demonstrated the upregulation of circFOXK2 in PDAC cells and...
primary tumors. Loss-of-function by both siRNA and shRNA and gain-of-function studies suggested the critical roles of circFOXK2 in PDAC progression by enhancing cell growth, migration, invasion, and metastasis in vitro and in vivo. Mechanistically, circFOXK2 functioned as a miRNA sponge and interacted with multiple proteins to regulate gene expressions.

Many studies have illustrated the importance of circRNAs in cancer progression (8–10). Previous circRNA profiling in 20 PDAC tissues identified 289 dysregulated circRNAs (21). Another study using 6 PDAC tissues discovered 351 differentially expressed circRNAs in PDAC (22). Recently, analysis using publicly available circRNA and miRNA datasets identified 256 differentially expressed circRNAs with...
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51 circRNA–miRNA interactions in PDAC (23). In general, these differentially expressed circRNAs were involved in protein localization, RNA binding, chromatin modification, and protein binding (21). circRNA_100782 functioned as a miR-124 sponge to promote PDAC cell proliferation via IL6-STAT3 pathway (24). hsa_circ_0006215 promoted migration via inhibiting miR-378a-3p in BxPC-3 cells (25). circ-PDE8A stimulated MACC1 expression by sponging miR-338 to promote cell invasion (26). However, the detailed roles of circRNAs in PDAC are still very unclear. Therefore, our study is the first to perform circRNA sequencing to identify differentially expressed circRNAs in PDAC. We identified 83 upregulated and 86 downregulated circRNAs in PDAC cells. Also, we demonstrated upregulation of circFOXK2 in PDAC cells and primary tumors played important roles in promoting cancer cell growth, migration, invasion, and liver metastasis.

The functional role of circRNAs was first described as miRNA sponges (6). CDR1as harbored 63 binding sites for miR-77. The interaction between CDR1as and miR-7 blocked the miR-7 activity. Since that, the functions of circRNAs as a "miRNA sponges" were comprehensively studied in many biological processes. Also, the importance of circRNA–miRNA interactions was also documented in many cancers, including PDAC. circLARP4 inhibited miR-424-5p and regulated LAST1 expression in gastric cancer (27). circITCH absorbed miR-17 and miR-224 to promote p21 and PTEN expression in bladder cancer (28). cirS-7 targeted miR-7 to promote EGFR/STAT3 signaling, while circMYM2 sponged miR335-5p and in turn favored the expression of JMJD2C in PDAC (29, 30). Here, we performed the bioinformatic analysis, luciferase assay, and miRNA pulldown assay to identify circFOXK2-interacting miRNAs in PDAC cells. We observed that circFOXK2 absorbed miR-942, and in turn promoted the expression of ANK1, GDNF, and PAX6. Studies indicated the potential of miR-942 as a diagnostic marker for PDAC (31), nevertheless, the roles of miR-942 in PDAC is poorly understood. We found that miR-942 inhibited PDAC cell growth and was the negative regulator of ANK1, GDNF, and PAX6, which were involved in PDAC cell growth, invasion, and metastasis (32–34).

Apart from functioning as miRNA sponge, there remained limited evidence on circRNA–protein interactions. circ-Amotl1 interacted with c-myc and promoted c-myc translocation to the nucleus in breast cancer (35). Circ-CTNNB1 interacted with DDX3 to transactivate YY1 and YY1 downstream targets in gastric cancer (36). Importantly, circRNA–protein interaction has not been documented in PDAC. Here, we developed a novel and highly specific circRNA-pulldown assay and identified the interacting proteins of circFOXK2 by mass spectrometry. We identified 94 circFOXK2-interacting proteins in PANC1 cells. Also, analysis using STRING database revealed that the circFOXK2-interacting proteins formed complexes with 101 protein–protein interactions. Gene ontology analysis indicated these proteins were associated with multiple biological processes, including cell adhesion, structural molecule activity, and mRNA splicing. These may suggest that circFOXK2 directly or indirectly interacts with multiple proteins with important roles in biological processes.

We then validated the results from mass spectrometry by RNA immunoprecipitation assay. Notably, we found that YBX1 and hnRNPK interacted with circFOXK2 in both PANC1 and CFPAC1 cells. YBX1 functions as RNA binding protein that regulates transcription, mRNA processing, and translation. YBX1 was frequently upregulated and involved in cell invasion and drug-resistance development in many cancers (37–39). In PDAC, studies reported that YBX1 promoted cell invasion (40). hnRNPK, as a partner of YBX1 in gene regulation, was also aberrantly expressed in various cancers (41). hnRNPK regulated cell migration via promoting the expression of GSN in lung adenocarcinoma (42). The upregulation of hnRNPK also favored cancer metastasis by promoting the expression of MMP-3 (43). Also, YBX1/hnRNPK complex was involved in cancer progression by regulating the splicing process (44). In this study, we found that YBX1 and hnRNPK regulated PDAC cell invasion. Also, we found that CORO1C, NUF2, PDXK, and PPP2R2C were directly regulated by YBX1 and hnRNPK complex in PDAC cells. Reported studies have demonstrated the growth-promoting role of NUF2 in cancer, including PDAC (45). Also, we observed the upregulation of NUF2 and PDXK in promoting PDAC cell growth and invasion. Importantly, among these YBX1 and hnRNPK targets, NUF2 and PDXK were also regulated by circFOXK2. Notably, knockdown of circFOXK2 reduced the interaction of YBX1 and hnRNPK to NUF2 and PDXK, suggesting circFOXK2 played an important role in YBX1- and hnRNPK-mediated gene regulation. Collectively, circFOXK2 complexed with YBX1 and hnRNPK to promote the expression of oncogenic proteins NUF2 and PDXK.

In conclusion, we profiled the circRNAs expressions in HPDE, PANC1, and SW1990 cells by circRNA sequencing. circFOXK2 was significantly upregulated in both PDAC cells and primary tumors. Gain-of-function and loss-of-function studies demonstrated important roles of circFOXK2 in promoting cancer cell growth, migration, invasion, and liver metastasis. We found that circFOXK2 functioned as a sponge of miR-942, and in turn promoted the expressions of ANK1, GDNF, and PAX6 (Fig. 7). More importantly, circFOXK2 interacted with YBX1 and hnRNPK to promote the expressions of oncogenic proteins NUF2 and PDXK (Fig. 7). These revealed a novel mechanism of circRNA in regulating gene expressions by interacting with YBX1/ hnRNPK complex.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: C.H. Wong, Y. Chen
Development of methodology: C.H. Wong, Y. Li
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.H. Wong, U.K. Lou, Y. Li, S.L. Chan, J.H.M. Tong, K.-F. To

Figure 7.
Schematic diagram describing the role of circFOXK2 in the development and progression of PDAC. circFOXK2 functioned as a sponge for miR-942 and in turn promoted the expression of ANK1, GDNF, and PAX6. Also, circFOXK2 interacted with YBX1 and hnRNPK to promote the expression of NUF2 and PDXK. These pathways favored cell growth, migration, invasion, and liver metastasis in PDAC.
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.H. Wong, Y. Li, Y. Chen

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Reference

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