**ZKSCAN1** gene and its related circular RNA (circZKSCAN1) both inhibit hepatocellular carcinoma cell growth, migration, and invasion but through different signaling pathways

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There is increasing evidence that circular RNA (circRNA) are involved in cancer development, but the regulation and function of human circRNA remain largely unknown. In this study, we demonstrated that ZKSCAN1, a zinc finger family gene, is expressed in both linear and circular (circZKSCAN1) forms of RNA in human hepatocellular carcinoma (HCC) tissues and cell lines. Here, we analyzed a cohort of 102 patients and found that expression of both ZKSCAN1 mRNA and circZKSCAN1 was significantly lower (P < 0.05) in the HCC samples compared with that in matched adjacent nontumorous tissues by reverse transcription PCR (RT-PCR). The low expression level of ZKSCAN1 was only associated with tumor size (P = 0.032), while the circZKSCAN1 levels varied in patients with different tumor numbers (P < 0.01), cirrhosis (P = 0.031), vascular invasion (P = 0.002), or microscopic vascular invasion (P = 0.002), as well as with the tumor grade (P < 0.001). Silencing both ZKSCAN1 mRNA and circZKSCAN1 promoted cell proliferation, migration, and invasion. In contrast, overexpression of both forms of RNA repressed HCC progression in vivo and in vitro. Silencing or overexpression of both forms of RNA did not interfere with each other. RNA-seq revealed a very different molecular basis for the observed effects; ZKSCAN1 mRNA mainly regulated cellular metabolism, while circZKSCAN1 mediated several cancer-related signaling pathways, suggesting a nonredundant role for ZKSCAN1 mRNA and circRNA. In conclusion, our results revealed two post-translational products (ZKSCAN1 mRNA and circZKSCAN1) that cooperated closely with one another to inhibit growth, migration, and invasion of HCC. circZKSCAN1 might be a useful marker for the diagnosis of HCC.

**Keywords**
circular RNA; HCC; qRT-PCR; RNA-seq; ZKSCAN1

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(Received 25 October 2016, revised 5 February 2017, accepted 6 February 2017, available online 17 March 2017)

doi:10.1002/1878-0261.12045

**Abbreviations**

AASLD, American Association for the Study of Liver Diseases; AFP, alpha-fetoprotein; circRNA, circular RNA; HCC, human hepatocellular carcinoma; hESCs, H1 human embryonic stem cells; MVI, microscopic vascular invasion; NCCN, National Comprehensive Cancer Network; RPKM, the reads per kilobase of exon model per million mapped reads; RT-PCR, reverse transcription PCR.
1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Statistics have shown that approximately 740 000 people are newly diagnosed with HCC and that this disease accounts for approximately 700 000 deaths annually worldwide (Marquardt et al., 2015). Approximately 55% of newly diagnosed patients were found in China, which consequently was regarded as the area with the highest prevalence of HCC (Torre et al., 2015). With the improvement of diagnostic approaches and treatment strategies, patients with HCC can be detected at an early stage and subjected to radical surgeries with a favorable prognosis. However, many patients (approximately 70% of newly diagnosed patients) with HCC missed using current diagnostic standards, and therefore, the best surgical opportunities were missed. Traditionally, alpha-fetoprotein (AFP) has been widely used for the clinical diagnosis of HCC. However, as a biomarker, AFP has limited clinical diagnostic value (especially for early diagnosis). Additionally, the 2010 guidelines of the American Association for the Study of Liver Diseases no longer recommend AFP for screening (Lin et al., 2015). To improve the therapeutic effect and prognosis of patients with HCC, it is imperative to search for more efficient biomarkers to increase the rate of early diagnosis of HCC. Therefore, a better understanding of the potential molecular mechanism underlying the occurrence and development of liver cancer, searches for new diagnostic markers and new targets of clinical treatment are of great important in liver cancer research.

Circular RNA (circRNA) are a special class of noncoding RNA that feature a covalently closed continuous loops (Jens, 2013). Compared with linear RNA, circRNA have the remarkable characteristic of noncanonical splicing without a free 3’ or 5’ end (Hentze and Preiss, 2013). circRNA were reported more than 20 years ago (Nigro et al., 1991), but have mostly been misinterpreted as splicing errors. Not until 2012 were circRNA rediscovered based on RNA sequencing (RNA-seq) data and shown to be widespread and diverse in eukaryotic cells. More than 20 000 different circRNA species have been reported in human and mouse tissues, and 700 in Caenorhabditis elegans. Typically, circular isoforms account for 5–10% of the total number of transcripts of their corresponding coding gene, but certain circRNA are up to 200 times more abundant than their linear counterparts (Hansen et al., 2013; Jeck et al., 2013; Salzman et al., 2013). Concerning their function, it is speculated that circRNA serve as epigenetic microRNA (miRNA) sponges (Hansen et al., 2013). In fact, previous studies have already validated this hypothesis (Hansen et al., 2013; Lasda and Parker, 2014). Recently, researchers have found that circRNA are involved in the development of several types of diseases, such as atherosclerosis and nervous system disorders (Burd et al., 2010; Chen and Old, 1990). Moreover, circRNA are becoming a new research hotspot in various cancers. For example, Hsa_circ_0001649 and hsa_circ_0005075 have been reported as potential diagnostic biomarkers for HCC, but how they regulate cancer progression at a mechanistic level is unclear (Qin et al., 2015). Additional studies are needed to investigate the role of circRNA and their potential diagnostic value in HCC.

A zinc finger with KRAB and SCAN domains 1 (ZKSCAN1, also named ZNF139) is a member of the zinc finger protein family. Members of the zinc finger protein family play regulatory roles for a variety of genes at the transcriptional level, have important effects on the maintenance of normal life activities, and are closely related to tumorigenesis, development, metastasis, and drug tolerance. van Dekken et al. (2009) found that increased ZKSCAN1 expression in adenocarcinoma at the esophagogastric junction was related to the proliferation of tumor cells. The ZKSCAN1 protein is overexpressed in certain gastroesophageal cancers (van Dekken et al., 2009) and has been suggested to regulate GABA type A receptor expression in the brain (Mulligan et al., 2012). However, to date, there have been no reports examining the relationship between ZKSCAN1 and HCC. Deep sequencing of the transcriptomes of numerous human cell lines, including HEK293, HeLa, and H1 human embryonic stem cells (hESCs), has revealed exon–exon junction reads connecting the 3’ end of exon 3 to the 5’ end of exon 2 (Jeck et al., 2013; Salzman et al., 2013). This finding suggests that exons 2 and 3 of ZKSCAN1 may be spliced together to form a covalently linked 668-nt circular RNA termed circZKSCAN1 (circBase ID: hsa_circ_0001727). circZKSCAN1 is particularly abundant in human brain and liver (Liang and Wilusz, 2014); however, its function in the regulation of HCC remains unknown.

In the present study, the role of ZKSCAN1 gene and its relative circRNA (circZKSCAN1) in the regulation of hepatocellular carcinoma cell growth, migration, and invasion and the potential mechanism were investigated.

Z. Yao et al.

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2. Materials and methods

2.1. Patients and tissue specimens

A total of 102 hepatocellular carcinoma tissue specimens and paired adjacent nontumorous tissues were collected from the hepatocellular carcinoma surgical specimens from February 2015 to February 2016 at The Second and The Third Affiliated Hospital of Sun Yat-sen University, China. After dissection, all tissue specimens were immediately preserved in RNA-fixer Reagent (Bieteke, Beijing, China) and stored at −80 °C until use. The corresponding adjacent nontumorous tissues were collected 5 cm from the edge of the cancer, as evaluated by an experienced pathologist. The histological grade was assessed following the National Comprehensive Cancer Network (NCCN) Clinical Practice Oncology Guidelines (V.1.2011). No radiotherapy, chemotherapy, or targeted therapy was conducted prior to surgery. All human studies were approved by The Institute Research Medical Ethics Committee of Sun Yat-Sen University. Written informed consent was obtained from all subjects.

2.2. Cell culture and lentivirus-mediated stable cell line construction

The human normal liver L02 cell line and the HCC cell lines (Huh7, SMMC-7721, BEL-7402, HepG2, and Hep3B) were purchased from Sun Yat-Sen University Laboratory Animal Center and maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% (v/v) FBS (Invitrogen, Carlsbad, CA, USA). After dissection, all tissue specimens were immediately preserved in RNA-fixer Reagent (Bieteke, Beijing, China) and stored at −80 °C until use. The corresponding adjacent nontumorous tissues were collected 5 cm from the edge of the cancer, as evaluated by an experienced pathologist. The histological grade was assessed following the National Comprehensive Cancer Network (NCCN) Clinical Practice Oncology Guidelines (V.1.2011). No radiotherapy, chemotherapy, or targeted therapy was conducted prior to surgery. All human studies were approved by The Institute Research Medical Ethics Committee of Sun Yat-Sen University. Written informed consent was obtained from all subjects.

2.3. RT-qPCR and RNase R treatment

Total RNA was isolated from cultured cells and fresh tissues using TRizol RNA isolation reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. The RNA integrity and concentration were determined using the Agilent RNA 6000 Nano Kit and Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Reverse transcription was performed using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and random primers. The primers were utilized as described in Table 1. All qPCR were performed using the GoTaq® qPCR Master Mix kit (Promega) according to the manufacturer’s protocol. The threshold cycle (Ct) value for each sample was calculated using the ABI analytical thermal cycler, and the relative RNA level was normalized to the GAPDH mRNA value. RNA

| Primer names | Sequences (5'→3') |
|--------------|------------------|
| HsacircZKSCAN1_F divergent | AGTCCCACTTCAACATTGTCT |
| HsacircZKSCAN1_R divergent | CACCTCACTATTACGATACATCC |
| HsacircZKSCAN1_F convergent | TACGCCCGATAGTGGAGA |
| HsacircZKSCAN1_R convergent | TGAAGTGGGACTGGGTGGC |
| HasZKSCAN1_F | TGTAATGAGTGCGGGAAGG |
| HasZKSCAN1_R | AATCAGGTATGAGTTTCGGTTG |
| HasGAPDH_F convergent | GAGTCAACGGATTTGGTCGT |
| HasGAPDH_R convergent | GACAAGCTTTCCCGATGTCG |
| HasGAPDH_F divergent | TCTTCACAGTTGCCATGTAGACC |
| HasGAPDH_R divergent | TGCCGGCTCAATTTATAGAAACCGG |

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expression data were calculated using the ΔΔCt method. Moreover, the RNase R digestion reaction was performed as previously reported (Jeck et al., 2013). Total RNA (5 μg) was incubated for 15 min at 37 °C with or without 3 U·μg⁻¹ of RNase R (Epigentech Biotechnologies, Madison, WI, USA). The RNA was subsequently purified by phenol–chloroform extraction and then subjected to RT-qPCR.

2.4. Western blotting

Western blotting was performed according to standard methods as described previously (Qi et al., 2015) using anti-ZKSCAN1 Abcam (Cambridge, MA, USA) and anti-GAPDH antibodies Santa Cruz (Santa Cruz, CA, USA). After washing, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) and visualized using enhanced chemiluminescence reagents (Forevergen Biosciences).

2.5. Cell proliferation, migration, and invasion assay

The [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] MTS viability assay (Promega) was performed according to standard methods as described previously (Luo et al., 2015). ZKSCAN1 and circZKSCAN1 knockdown or overexpression cell lines and control cells were seeded at 2 × 10⁴ cells in 100 μL medium per well of a 96-well plate Corning Incorporated (Corning, NY, USA) for the MTS assay (Qi et al., 2014; Qin et al., 2016). The cells were incubated for a total of 3 days with fresh culture medium. Proliferation was analyzed using the MTS reagent at day 1, day 2, and day 3. Triplicate samples were evaluated for each treatment.

For the invasion assay, the transwell system and Matrigel BD Biosciences (New York, NY, USA) were used according to the manufacturers’ protocols and the invasion assay was performed according to the methods as described previously (Qi et al., 2014; Qin et al., 2016). Aliquots of 1 × 10⁶ cells were seeded into the upper chambers without a Matrigel coating. The rest of the assay was conducted as described for the invasion assay. Cell numbers in five high-power fields were counted after crystal violet staining. Three biological replicates were conducted.

2.6. Xenografts in mice

Twenty-five (n = 5 per group) 6-week-old male nude mice were purchased from Sun Yat-Sen University Laboratory Animal Center. Control (negative cells) and treated cells (cells transduced with the indicated lentivirus vector) were diluted to a concentration of 1 × 10⁶ mL⁻¹ in physiological saline. Mice were injected subcutaneously with 0.1 mL of the suspension into the back flank. When a tumor was palpable, tumor growth was measured every week with a caliper. After five weeks, the mice were killed, and the tumors were dissected and weighed. The tumor volume (V) was calculated according to the following formula: V = L × W² × 0.5 (L, length; W, width). All animal studies were approved by The Institute Research Medical Ethics Committee of Sun Yat-Sen University.

2.7. Immunohistochemistry and fluorescence in situ hybridization (FISH)

Immunohistochemistry was performed according to the standard methods as described previously (Yi et al., 2016). Briefly, paraffin-embedded tissues were stained with anti-ZKSCAN1 antibody (1 : 100 dilution; Abcam) at 4 °C overnight. Rabbit IgG was used as a negative control. After washing, the slides were treated with rabbit biotinylated secondary antibody at room temperature for 30 min. The slides were then stained with the ABC Elite kit Vector Labs (Burlingame, CA, USA). Finally, the slides were counterstained with hematoxylin, dehydrated, cleared, and then mounted with Permount mounting medium Fisher Scientific (Fair Lawn, NY, USA).

For the FISH assay, PCR fragments containing the T7 promoter were amplified using specific primers for the back splice region of circZKSCAN1. RNA probes were transcribed using the TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific, Rochester, NY, USA) with the corresponding PCR product as a template for transcription, and they were labeled with Alexa Fluor 546 using the ULYSIS Nucleic Acid Labeling Kit (Invitrogen) according to the manufacturer’s protocol. HepG2 cells were grown to 50–75% confluence at the
time of fixation. After prehybridization, the cells were hybridized to Alexa Fluor 546-labeled probes specific to circZKSCAN1 at 60 °C overnight. Signals were detected under a microscope (Olympus Corp.). Primers for FISH analysis were Hsacirc cZKSCAN1_P1-FISH: TAAT ACGACTCATAAGGGAGTCCCACCTCAACA TTCGTCT, HsacircZKSCAN1_P2-FISH: CACCTTC ACTATTAGATACCATCC.

2.8. RNA-seq

Following RNA isolation, the RNA was quantified (Qubit RNA Assay Kit; Life Technologies, Inc.), and the quality was assessed (RNA6000 Nano Kit and BioAnalyzer 2100; Agilent). Next, 1000 ng was used as the input for the VAHTSTM mRNA-seq v2 Library Prep Kit for Illumina® (Vazyme, Inc., Piscataway, NJ, USA), and sequencing libraries were created according to the manufacturer’s protocol. Briefly, poly(A) RNA was purified via two rounds of hybridization to Dynal Oligo (dT) beads. Poly (A)+ RNA was fragmented and then used for first- and second-strand cDNA synthesis with random hexamer primers. The cDNA fragments were treated with the End-It DNA EndRepair Kit to repair the ends, then modified with Klenow to add an A at the 3’ end of the DNA fragments, and finally ligated to the adapters. The ligated cDNA products were subjected to PCR amplification. The library quality was determined using a Bioanalyzer 2100 (Agilent). The RNA-seq libraries were sequenced using the Illumina HiSeq 4000 platform. Reads were aligned to the human reference genome GRCh37/hg19 using tophat v2.1.0 (Langmead, 2012). The reads per kilobase of exon model per million mapped reads (RPKM) of each gene was calculated based on the length of the gene and the read counts mapped to the gene. Differential expression analysis of two conditions/groups (two biological replicates per condition) was performed using the previously described statistical model (Audic and Claverie, 1997). The resulting P-values were adjusted using Benjamini and Hochberg’s approach for controlling the false discovery rate. Genes with an adjusted P-value < 0.05 were considered differentially expressed. We used kobas software (Peking University, Beijing, China) to test the statistical enrichment of the differentially expressed genes in the KEGG pathway. circRNA were used as seeds to enrich the circRNA–miRNA–gene network according to the analysis of miRanda (http://www.microrna.org/) combined with our RNA-seq data. Cytoscape (http://www.cytoscape.org/) was applied to build a circRNA–miRNA–mRNA interaction network, according to a previous report (Shang et al., 2016).

2.9. Statistics

Data are presented as the mean ± SE. Statistical analyses were conducted with the srs11.5 statistical software package. The primary statistical test was one-way ANOVA. Differences in the expression of ZKSCAN1 and circZKSCAN1 between different clinical parameters of HCC were analyzed using the Student’s t-test. P < 0.05 was considered statistically significant. The receiver-operating characteristic (ROC) is a comprehensive index that is used to reflect the sensitivity and specificity of continuous variables. A larger area under the curve reflects a higher diagnostic value of the variable. To evaluate whether ZKSCAN1 and circZKSCAN1 could serve as a potential marker for the diagnosis of HCC, the ROC curve was constructed, and the AUC value was calculated to explore the diagnostic value of ZKSCAN1 and circZKSCAN1.

3. Results

3.1. ZKSCAN1 was down-regulated in human HCC and a circular form of its RNA, circZKSCAN1, was validated

To investigate the expression patterns of ZKSCAN1, HCC tissues and adjacent nontumorous tissues were subjected to reverse transcription PCR (RT-PCR) and western blot analysis, and the results are shown in Fig. 1A. Both the mRNA and protein of ZKSCAN1 were significantly down-regulated, indicating that the ZKSCAN1 gene may be involved in the progression of HCC. RT-PCR assays were used to verify this circular form of ZKSCAN1. The assays performed with divergent primers indicated that circZKSCAN1 was expressed in the samples of HCC tissues (Fig. 1B). Moreover, Sanger sequencing of the RT-PCR products amplified by divergent primers further confirmed the back splice junction of circZKSCAN1 (Fig. 1C). To further confirm the circular characteristics of circZKSCAN1, we used the enzyme RNase R, a highly processive 3’ to 5’ exoribonuclease that does not act on circular RNA (Vincent and Deutscher, 2009). As expected, in contrast to the linear control gene GAPDH, the circular RNA was resistant to RNase R treatment (Fig. 1D), demonstrating that in addition to the ZKSCAN1 mRNA, there was a naturally circular RNA form of the ZKSCAN1 gene.
3.2. The expression and potential diagnostic values of ZKSCAN1 and cirZKSCAN1 in human HCC

In this study, differential expression of ZKSCAN1 and cirZKSCAN1 was observed based on qRT-PCR analysis of 102 HCC tissue samples and paired adjacent non-tumorous liver tissues. The GAPDH gene served as the internal standard. Both linear ZKSCAN1 and cirZKSCAN1 were significantly down-regulated in HCC samples (Fig. 2A, B). Further determination of the relationship between the expression of ZKSCAN1, cirZKSCAN1, and the clinical parameters of HCC showed that ZKSCAN1 and cirZKSCAN1 expression was associated with several clinicopathological features. As shown in Table 2, of all the clinical parameters, a low expression level of ZKSCAN1 was only associated with a tumor size less than 5 cm ($P = 0.032$). Additionally, the cirZKSCAN1 levels varied in patients with different tumor numbers ($P < 0.01$), cirrhosis ($P = 0.031$), vascular invasion ($P = 0.002$), or microscopic vascular invasion (MVI, $P = 0.002$), as well as with the tumor grade ($P < 0.001$). As shown in Fig. 2C, the area under the ROC curve (AUC) of cirZKSCAN1 was 0.834, with a sensitivity of 82.2% and specificity of 72.4%, which is much higher than the mRNA of ZKSCAN1 (AUC = 0.474) (Fig. 2C), indicating that cirZKSCAN1 expression could serve as a biomarker for distinguishing cancerous tissue from adjacent noncancerous liver tissue.

3.3. ZKSCAN1 and cirZKSCAN1 were down-regulated in HCC cell lines and did not affect one another’s expression

To analyze the potential function of ZKSCAN1 and cirZKSCAN1 in HCC cells, we next investigated ZKSCAN1 mRNA and cirZKSCAN1 expression in some human HCC cell lines. Five HCC cell lines (Huh7, SMMC-7721, BEL-7402, HepG2, and Hep3B) and a control group (human normal liver cell line, L02) were assessed by qRT-PCR. As shown in Fig. 3A, B, the ZKSCAN1 mRNA and cirZKSCAN1 transcripts were down-regulated in all HCC cell lines compared with the control group. To further investigate the role of ZKSCAN1 in HCC, we chose two cell lines, HepG2 and SMMC-7721, which demonstrated the lowest expression levels of both forms of ZKSCAN1 RNA, to establish stable cell lines. We confirmed the efficient silencing and ectopic expression by qRT-PCR. In addition, our results indicated that over-expression or knockdown treatments of ZKSCAN1 or cirZKSCAN1 could effectively increase or decrease their mRNA expression levels but did not interfere...
with their interaction with one another in SMMC-7721 cells and HepG2 cells (Fig. 3C,D).

3.4. Influence of overexpression and knockdown of ZKSCAN1 and cirZKSCAN1 on proliferation, migration, and invasion of HCC cells

To test whether ZKSCAN1 mRNA and cirZKSCAN1 could affect the biological behavior of HCC cells, we performed gain-of-function and loss-of-function assays in SMMC-7721 cells and HepG2 cells. We assessed the effects of the two RNA on the proliferation of HCC cells. The results of the MTS assays revealed increased proliferative ability in ZKSCAN1 mRNA and cirZKSCAN1 SMMC-7721-knockdown cells, while a sharp reduction in the proliferation rate was observed in SMMC-7721-overexpressing cells (Fig. 4A). The same conditions were detected in HepG2 cells (Fig. 4B). The results suggested that knockdown of ZKSCAN1 or cirZKSCAN1 could effectively accelerate cell proliferation. In contrast, overexpression of ZKSCAN1 or cirZKSCAN1 effectively inhibited cell proliferation. The Boyden chamber and migration through a polycarbonate membrane showed that overexpression of ZKSCAN1 or cirZKSCAN1 reduced cell migration (Fig. 4C,E) and cell invasion (Fig. 4D,F). In contrast, suppression of ZKSCAN1 or cirZKSCAN1 had the reverse effect on invasion (Fig. 4C,E) and cell migration (Fig. 4D,F). These experiments showed that overexpression and knockdown in SMMC-7721 cells provided more significant results, and thus, SMMC-7721-based cell lines were used in subsequent studies.

3.5. Overexpression and knockdown of ZKSCAN1 or cirZKSCAN1 modulated tumor growth in vivo

As shown in Fig. 5, SMMC-7721 cells with overexpressed ZKSCAN1 or cirZKSCAN1 were subcutaneously injected into the back flank of nude mice. Our results showed that the growth of tumors from overexpressed ZKSCAN1 or cirZKSCAN1 xenografts was significantly inhibited compared with that from the control xenografts. In contrast, the growth of tumors...
from knockdown ZKSCAN1 or cirZKSCAN1 xenografts was significantly accelerated compared with that from the control xenografts.

### 3.6. Cellular localization

The function of a gene is closely related to its intracellular localization. As a member of the zinc finger protein family, ZKSCAN1 protein has been reported to be located in the nucleus of gastric cancer tissues and to play regulatory roles affecting a variety of genes at the transcriptional level (Fan et al., 2015). Immunohistochemistry analysis using an anti-human ZKSCAN1 antibody was used to examine its protein expression level and cellular localization in the normal and HCC liver tissues. Surprisingly, our results revealed relatively stronger, discrete, and granular cytoplasmic staining in the normal liver samples (Fig. 6A). In contrast, little ZKSCAN1 was expressed in HCC liver tissues. In addition,
information concerning the location of ZKSCAN1 in the human protein atlas (http://www.proteinatlas.org/) showed that the majority of malignant gliomas, prostate and colorectal cancers, as well as most other malignancies, showed moderate to strong staining of ZKSCAN1, which was located in the nucleus, but only weak or negative cytoplasmic staining in liver cancer, which was consistent with our results. Furthermore, data from the database mentioned above showed that ZKSCAN1 was most likely located in mitochondria in certain cell lines, which indicated that ZKSCAN1 may play a transcription-independent role in mitochondria in HCC. Circular RNA with different origins may be located in different parts of the cell, with circRNA originating from exons located mainly in the cytoplasm while those originating from introns are located in the nucleus. FISH analysis showed that circZKSCAN1 was most likely located in the cytoplasm (Fig. 6B,C), consistent with previous studies (Liang and Wilusz, 2014), and it might be involved in the regulatory network of the cell.

Fig. 3. qRT-PCR analysis of the expression of ZKSCAN1 and circZKSCAN1 with and/or without interferences in human HCC cell lines. (A) The expression levels of ZKSCAN1 in five HCC cell lines (Huh7 cell line, SMMC-7721 cell line, BEL-7402 cell line, HepG2 cell line, and Hep3B cell line) were significantly lower than those in the human normal hepatic L02 cell line. **P < 0.001. (B) The expression levels of circZKSCAN1 in five HCC cell lines (Huh7 cell line, SMMC-7721 cell line, BEL-7402 cell line, Hep3B cell line, and HepG2 cell line) were significantly lower than those in the L02 cell line. **P < 0.001. (C) The expression levels of ZKSCAN1 and circZKSCAN1 in the control, overexpression, and knockdown of circZKSCAN1 or ZKSCAN1 SMMC-7721 cell line. (D) The expression levels of ZKSCAN1 and circZKSCAN1 in the control, overexpression, and knockdown of circZKSCAN1 or ZKSCAN1 in the HepG2 cell line.

Fig. 4. Cell proliferation, invasion, and migration of the SMMC-7721 cell line and HepG2 cell line following the overexpression and knockdown of circZKSCAN1 or ZKSCAN1. (A) MTS method examining SMMC-7721 and HepG2 (B) cell proliferation of five different treatments on days 1, 2, and 3. (C) Migration assay and invasion assay (D) of SMMC-7721 cells. Migration assay (E) and invasion assay (F) of HepG2 cells transfected with different treatments. Images are representative of the cells invading one field. **P < 0.001. Control: negative control; sh-circ: knockdown treatment of circZKSCAN1; over-circ: overexpression treatment of circZKSCAN1; sh-linear: knockdown treatment of ZKSCAN1; over-linear: overexpression treatment of ZKSCAN1.
Z. Yao et al.
circZKSCAN1 and hepatocellular carcinoma

A

B

C

D

E

F

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3.7. RNA-seq revealed a different molecular basis for ZKSCAN1 mRNA and circZKSCAN1 in SMMC-7721 cells

To explore the underlying mechanism by which ZKSCAN1 regulated cell proliferation and invasion in HCC cells, we performed RNA-seq analysis to identify changes in gene expression after knockdown of ZKSCAN1 mRNA (shZKSCAN1) or circZKSCAN1 (shcircZKSCAN1) in SMMC-7721 cells. We detected 372 differentially expressed genes in SMMC-7721-knockdown cells (shcircZKSCAN1) compared with the negative control SMMC-7721 cells (control). Additionally, there were 346 differentially expressed genes in SMMC-7721 cells following the knockdown of ZKSCAN1. There were 166 overlapping differentially expressed genes in both of the sets mentioned above, which suggested that there were significant differences between the shZKSCAN1 and shcircZKSCAN1 treatments (Fig. 7A). Moreover, the genes that were significantly differentially expressed were also captured in the heat map (Fig. 7B). To further assess the biological insight gleaned from the transcript-level response to the loss of circZKSCAN1, we performed KEGG
enrichment analysis. The differentially expressed genes were more likely to be enriched in the PI3K pathway, migration pathway, actin cytoskeleton pathway, adhesion pathway, and cytokine interaction pathway, among others, following the knockdown of cir-ZKSCAN1 (Fig. 7C). However, the differentially expressed genes following the knockdown of ZKSCAN1 were mainly enriched in metabolic pathways (Fig. 7D).

3.8. qRT-PCR analysis of ZKSCAN1- and cir-ZKSCAN1-related downstream genes in SMMC-7721 cells

We validated the ZKSCAN1- and cir-ZKSCAN1-related downstream genes in SMMC-7721 cells by qRT-PCR analysis (Fig. 8). AKR1B10, CYP1A1, ACSL1, and ALPP, which were related to cellular metabolism, were synchronously up-/down-regulated with the knockdown and overexpression of ZKSCAN1, but not cir-ZKSCAN1. Similar expression patterns were detected in apoptosis and migration-related genes, including VEGFA, ENO2, FN1, and VTN. The expression levels of RAC2, EFNA3, and caspase 3, which are important factors in the intrinsic apoptosis pathway, were only affected by knockdown and overexpression treatments of cir-ZKSCAN1. Similar expression patterns were detected in cell proliferation-related genes, including TGFB1, ITGB4, CXCR4, survivin, and CCND1. However, there was also an interaction between cir-ZKSCAN1 and ZKSCAN1, and the expression levels of COL3A1, CDH5, MYB, PDK1, and BCL2 were regulated by both cir-ZKSCAN1 and ZKSCAN1.

The above results clearly indicated that the two mature RNA transcripts from the same ZKSCAN1 gene may play different roles in HCC cells. We propose that after translation, ZKSCAN1 protein is translocated to mitochondria and plays a regulatory role in metabolism, further impacting cell death and metastasis. cir-ZKSCAN1 may function as a ceRNA, which is regulated by other RNA by competing for the same pool of miRNA, playing a role in proliferation- and invasion-related signaling pathways. The detailed regulatory mechanism requires further investigation.

4. Discussion

ZKSCAN1 had been reported to be up-regulated in gastric cancer (Bartel, 2009). However, few reports have examined the role of ZKSCAN1 in HCC, and little is known about its molecular basis. Through a series of functional experiments in the present study, we showed that, in addition to ZKSCAN1 mRNA, a circular RNA, cir-ZKSCAN1, was present in HCC tissues and cells. The expression levels of cir-ZKSCAN1 and ZKSCAN1 were both down-regulated in HCC cell lines. However, there was no obvious relationship between ZKSCAN1 and cir-ZKSCAN1 expression. The potential diagnostic analysis suggested that cir-ZKSCAN1 had the maximum value compared with ZKSCAN1. Knockdown treatment of ZKSCAN1 or cir-ZKSCAN1 effectively accelerated cell proliferation, migration, and invasion. In contrast, overexpression of ZKSCAN1 or cir-ZKSCAN1 effectively inhibited cell proliferation, migration, and invasion. Additionally, the growth of tumors from knockdown ZKSCAN1 or cir-ZKSCAN1 xenografts was significantly accelerated compared with the control xenografts. ZKSCAN1, as a member of the zinc finger protein family, is a transcription factor and thus theoretically was more likely to be located in the nucleus. However, most of the ZKSCAN1 protein was located in the cytoplasm, and more often in mitochondria in the normal liver tissues. FISH analysis of the location of cir-ZKSCAN1, which was derived from exons, showed that it was most likely located in cytoplasm, which is consistent with previous results (Jecck and Sharpless, 2014). Furthermore, significant differential expression was observed in downstream genes following the knockdown of ZKSCAN1 or cir-ZKSCAN1 compared with the negative control cells in the RNA-seq analysis. KEGG analysis suggested that these differentially expressed genes after cir-ZKSCAN1 silencing were more likely to be enriched in the PI3K pathway, migration pathway, actin cytoskeleton pathway, adhesion pathway, and cytokine interaction pathway, among others. However, the differentially expressed genes were enriched in metabolic pathways following the knockdown of ZKSCAN1. qRT-PCR analysis suggested that the expression levels of cell metabolism, apoptosis, migration, and cell proliferation-related genes were consistent with the RNA-seq data. Therefore, cir-ZKSCAN1 and ZKSCAN1 could play important regulatory roles in different signaling pathways.

Based on our previous studies, cir-ZKSCAN1 and ZKSCAN1 are involved in the progression of HCC, as shown in Fig. S1. The ZKSCAN1 gene can generate two types of RNA, including ZKSCAN1 mRNA and cir-ZKSCAN1. ZKSCAN1 mRNA can be translated into a functional protein, which is likely to be located in mitochondria and appears to play an important role in maintaining the normal metabolism of HCC cells. The metabolism-facilitated proteins CYP1A1, ACSL1, ALPP, and AKR1B10 were factors downstream of
ZKSCAN1 protein. Metabolic disorders further impacted cell death and metastasis, in part by regulating FN1, VTN, ENO2, and VEGFA. Additionally, circZKSCAN1 may have acted as a competitive inhibitor to retain the endogenous RNA and to regulate the expression of tumor cell proliferation and metastasis-related genes, including the apoptotic genes RAC2, EFNA3, and caspase 3, and the cell proliferation-related genes TGFβ1, ITGB4, CXCR4, survivin, and CCND1. We assumed that circZKSCAN1 acted as a ceRNA to regulate its circRNA–miRNA–mRNA network and that the interactions could be predicted by TargetScan, miRanda, and our RNA-seq data. Based on these analyses, a total of eight miRNA and 87 mRNA were predicted to interact with circZKSCAN1 (Fig. S2). RNA pull-down and other experiments are needed to further elucidate the mechanism of circZKSCAN1. Thus, the two ZKSCAN1 gene post-translational products (ZKSCAN1 mRNA and circRNA) play important roles in the development of human HCC cells, acting independently and cooperating closely with one another to promote cancer growth.

5. Conclusions

In summary, we demonstrated the expression profiles of circZKSCAN1 and ZKSCAN1 in the human HCC tissues and cell lines. CirZKSCAN1 had greater

Fig. 8. qRT-PCR analysis of the expression of 21 genes selected from the RNA-seq results. Control: normal SMMC-7721 cell line; sh-circ: knockdown treatment of circZKSCAN1 in the SMMC-7721 cell line; over-circ: overexpression treatment of circZKSCAN1 in the SMMC-7721 cell line; sh-linear: knockdown treatment of ZKSCAN1 in the SMMC-7721 cell line; over-linear: overexpression treatment of ZKSCAN1 in the SMMC-7721 cell line. The y-axis shows the gene expression levels after normalization to the reference gene GAPDH. *P < 0.05, compared with NC.
potential value for the diagnosis of HCC compared with ZKSCAN1. Furthermore, overexpression and knockdown treatments of cirZKSCAN1 and ZKSCAN1 effectively influenced the proliferation, invasion, and migration of human HCC cells. In addition, ZKSCAN1 protein was located in the cytoplasm, especially in the mitochondria in the normal liver tissues. As an exonic circRNA, cirZKSCAN1 was located in the cytoplasm. The downstream signaling pathways were significantly differentially expressed following the knockdown of ZKSCAN1 or cirZKSCAN1 compared with the negative control cells in the RNA-seq analysis. A putative pathway for cirZKSCAN1 and ZKSCAN1 suggested that the ZKSCAN1 gene could be involved in the metabolism, apoptosis, proliferation, and metastasis of HCC. The results presented herein suggest that cirZKSCAN1 may function as a diagnostic biomarker of HCC and may provide a novel understanding of the mechanisms associated with human HCC.

Acknowledgements
This study was funded by National Natural Science Foundation of China (815711144, 81570593); Science and Technology Planning Project of Guangdong Province, China (2014A020212122); Medical Research Foundation of Guangdong Province, China (2015A010119103); the Innovative Funds for Small and Medium-Sized Enterprises of Guangdong Province (2015A010119103); Cultivate Project for the Interdiscipline and Emerging Disciplines by Sun Yat-sen University (15ykjc19c); and the Innovative Funds for Small and Medium-Sized Enterprises of Guangdong Province (2016A010119103).

Author contributions
ZY, JL, BL, and YY conceived and designed the project; KH, JL, and HH acquired the data; QW, PZ, ZX, CH, and ZH analyzed and interpreted the data; ZY and JL wrote the manuscript.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. The potential molecular mechanism of ZKSCAN1 and circZKSCAN1 in the progression of HCC cancer growth.

Fig. S2. The predicted circZKSCAN1-targeted circRNA–miRNA–mRNA/gene network based on the RNA-seq data.