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ZNF204P is a stemness-associated oncogenic long non-coding RNA in hepatocellular carcinoma

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Hepatocellular carcinoma is a major health burden, and though various treatments through much research are available, difficulties in early diagnosis and drug resistance to chemotherapy-based treatments render several ineffective. Cancer stem cell model has been used to explain formation of heterogeneous cell population within tumor mass, which is one of the underlying causes of high recurrence rate and acquired chemoresistance, highlighting the importance of CSC identification and understanding the molecular mechanisms of CSC drivers. Extracellular CSC-markers such as *CD133*, *CD90* and *EpCAM* have been used successfully in CSC isolation, but studies have indicated that increasingly complex combinations are required for accurate identification. Pseudogene-derived long non-coding RNAs are useful candidates as intracellular CSC markers - factors that regulate pluripotency and self-renewal − given their cancer-specific expression and versatile regulation across several levels. Here, we present the use of microarray data to identify stemness-associated factors in liver cancer, and selection of sole pseudogene-derived lncRNA *ZNF204P* for experimental validation. *ZNF204P* knockdown impairs cell proliferation and migration/invasion. As the cytosolic *ZNF204P* shares miRNA binding sites with *OCT4* and *SOX2*, well-known drivers of pluripotency and self-renewal, we propose that *ZNF204P* promotes tumorigenesis through the miRNA-145-5p/*OCT4*, *SOX2* axis. [BMB Reports 2022; 55(6): 281-286]

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the main form (90%) of primary liver cancer (1), which is the fourth most common cause of cancer-related death worldwide. Cancer stem cell (CSC) model has been attributed to create tumor heterogeneity, in which a sub-set of tumor cells capable of self-renewal and extensive proliferation result in high recurrence rates and chemoresistance acquisition (2). CSC-induced tumor heterogeneity is associated with poor prognosis in several cancers (3) and thus CSC-specific markers (extracellular and intracellular) have been reported as crucial therapeutic targets (4) to selectively eradicate cells with tumorigenic capabilities. As CSCs share their key properties such as self-renewal and proliferation with normal stem cells, there is an unsurprising large overlap between CSC and normal stem cell surface markers such as *CD133*, *CD90* and *EpCAM* (5). However, studies indicate that detailed curation is still required to fully distinguish CSCs (4). Transcription factors *OCT4* and *SOX2*, best defined as essential players in maintaining pluripotency and self-renewal in embryonic stem cells/induced pluripotent stem cells (6), can be reactivated in CSCs showcasing similar properties (7), albeit under an aberrant light. Identification of such stemness-related factors would be useful in addressing CSCs-related malignant phenotypes (4).

ROR, HOTAIR and UCA1 are some well-established CSC-regulating long non-coding RNAs (lncRNAs) through various molecular mechanisms involving proliferation, self-renewal and metastasis promotion (8). lncRNAs are ideal target factors as their regulation span epigenetic, post-transcriptional and transcriptional levels (9) with reported function in various cancers (10) and cancer-type specific expression (11). Pseudogenes which were first defined as 'junk' genomic loci, have recently undergone resuscitation as a novel lncRNA class, namely pseudogene-derived lncRNAs with high relevance in cancer initiation and progression (12). Pseudogene-lncRNAs such as *PTENP1* (13) and *DUXAP10* (12) have demonstrated their critical role as competing endogenous RNAs by binding competitively to shared microRNAs (miRNAs) that target either their cognate gene or unrelated genes (14). Thus, identifying stemness-related factors in form of lncRNAs would

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be highly relevant in deepening understanding of gene circuits regulating cancer stemness.

In our study, we propose a user-friendly method of using public microarray data to identify lncRNA stemness-related factors in liver cancer, followed by preliminary experimental validation. Our candidate *ZNF204P* is a pseudogene-derived lncRNA and its knockdown inhibits cell proliferation, migration/invasive and colony formation properties. The cytosolic *ZNF204P* shares a miRNA-binding site (miRNA-145-5p) with *OCT4* and *SOX2*, implying its role as a molecular decoy for tumor-suppressive miRNA-145-5p by interfering with its targeted degradation of *OCT4* and *SOX2*. To the best of our knowledge, this study is the first to publish experimental validation of *ZNF204P*, and our date collectively indicates that *ZNF204P* is a stemness-associated oncogenic lncRNA in HCC with possible function as a miRNA-decoy, providing another avenue to identify prognostic and/or or therapeutic targets.

RESULTS

Stemness-related candidate selection

Microarray datasets (GSE50206, GSE25097) were utilized for stemness-related candidate selection. HCC samples from GSE25097 were selected based on elevated expression of various stemness markers (*EpCAM*, *Sall4*, *BMI-1* and *SOX12*) (15-17). Up-regulated genes in embryonic stem cells (ESCs) relative to human dermal fibroblast (HDFs) (Fig. 1A, left) and those in selected HCC samples compared to healthy samples (Fig. 1A, right, Supplementary Table 1) were shortlisted through GEO2R analysis. log2FC-filtered significantly dysregulated genes were overlapped to find common gene candidates (Fig. 1B, Supplementary Fig. 1A, B) that regulate stemness in both normal stem cells and tumor samples. Gene ontology (GO) analysis of resulting 139 genes presented association with stemness regulation and maintenance (Fig. 1C). We focused on *ZNF204P*, the sole pseudogene-derived lncRNA given its importance as a growing novel class of lncRNA in cancer stemness regulation (Supplementary Table 1). We found that *ZNF204P* is up-regulated in tumor samples (Fig. 1D) with poor prognosis (Fig. 1E). Similar analysis patterns are reflected in well-known stemness-driven tumorigenic factors (18, 19) MYCN and MYB (Supplementary Fig. 2). Furthermore, gene set enrichment analysis depicted enrichment for increased cell proliferation and maintenance of stem cells in *ZNF204P*-high group (Fig. 1F), consolidating our stand that *ZNF204P* is a stemness-associated oncogenic lncRNA.

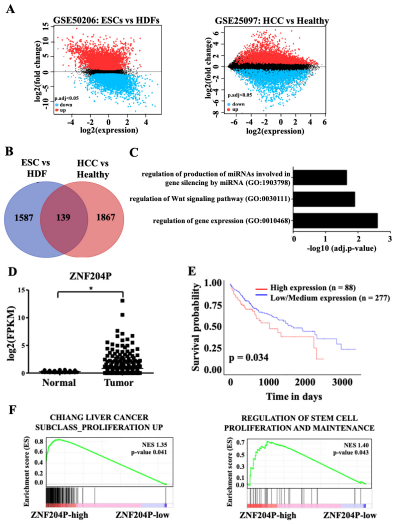

Fig. 1. Selection of *ZNF204P* as a stemness-associated pseudogene-derived lncRNA. (A) Dysregulated genes within human embryonic stem cells (ESCs)/human dermal fibroblasts (HDFs) (left) and hepatocellular carcinoma (HCC)/healthy samples (right) visualized in mean difference plot (P-value < 0.05). (B) 139 genes commonly dysregulated between ESCs/HDF and HCC/Healthy groups. (C) GO analysis demonstrates involvement of common genes in biological processes that enhance tumorigenesis. (D) Tumor-specific upregulation of *ZNF204P* implies oncogenic role. (E) Poor survival outcome of high-expression groups (right). *P < 0.05 (two-tailed unpaired t-test analysis); ns indicates no significance. (F) Gene sets for increased proliferation in cancer cells (left) and stem cell maintenance (right) are enriched in *ZNF204P*-high group.

ZNF204P knockdown impairs cell proliferation, cell migration and invasion in HepG2 cell line

FANTOM CAT browser was accessed to define transcription start site (TSS) of *ZNF204P* (Fig. 2A) for CRISPR interference (CRISPRi) single-guide RNA (sgRNA) design. We found that most of the FANTOM-annotated *ZNF204P* transcripts corresponded

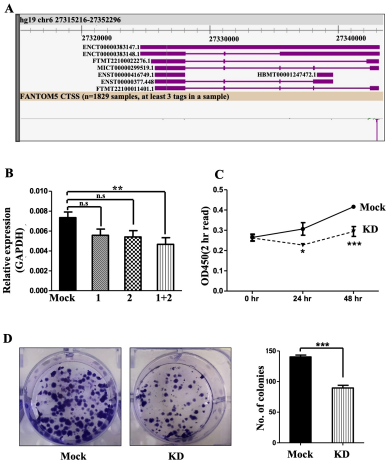

Fig. 2. Knockdown of *ZNF204P* inhibits cell proliferation. (A) Graphical representation of *ZNF204P* transcripts corresponding to same CAGE cluster based on CAGE tag starting sites (CTSS). (B) Combined use of 2 sgRNAs showed the best KD efficacy. (C) *ZNF204P* KD led to reduced cell proliferation over time. (D) Reduced number of colonies were observed post-*ZNF204P* KD in colony formation assay (left) and quantification of colonies in an average of 3 fields per group (right). All data were acquired from three independent experiments (n = 2). *P < 0.05; **P < 0.01; ***P < 0.001 (two-tailed unpaired t-test analysis); ns indicates no significance.

to the same CAGE (Cap Analysis of Gene Expression) cluster (Fig. 2A) and combination of 2 custom-designed sgRNAs produced the highest knockdown (KD) efficacy relative to the control group (Fig. 2B) and was thus used in all subsequent KD experiments.

In vitro cell survival assays in form of Cell-counting Kit-8 showed that *ZNF204P* KD significantly inhibits cell survival (Fig. 2C) and number of colonies were drastically reduced in KD groups (Fig. 2D). Next, we examined the effect of *ZNF204P* KD on tumor cell migration through Transwell and wound healing assays. Following knockdown of *ZNF204P*, both migratory (Fig. 3A) and invasive (Fig. 3B) abilities of HepG2 were significantly weakened compared to control groups. *ZNF204P* regulation over cell migration was further substantiated when inhibited wound closure presented in the knockdown group relative to the control group (Fig. 3C). Altogether, our data presents *ZNF204P* as an oncogenic lncRNA that plays a critical role in cell proliferation, self-renewal and migration/invasion in HepG2 cells.

ZNF204P as a predicted miRNA-145 sponge

Fractionated RT-qPCR with *MALAT1* and *DANCR* as positive controls established *ZNF204P* as a cytosolic lncRNA (Fig. 4A). Since the main mechanism of action of pseudogene-lncRNAs is through competing endogenous RNAs (ceRNAs), whereby lncRNAs compete for miRNAs that bind to cognate or unrelated genes, we first examined the relationship between *ZNF204P* and its parental gene *ZNF79*. As no shared miRNA sites were detected (Supplementary Fig. 3A, Supplementary Table 4), we investigated the mode of function of *ZNF204P* in relation to well-known regulators of stemness in both normal and cancer stem cells – *OCT4*, *SOX2* and *Nanog*. miRNA-145-5p stood out within the predicted 7 shared miRNAs between *ZNF204P* and different combinations of *OCT4*/*SOX2*/*Nanog* (Fig. 4B, Supplementary Table 4) as its tumor suppressor role by degrading *OCT4* and *SOX2* has been covered previously (20, 21). Based on confirmation of down-regulated expression of miRNA-145-5p in TCGA-LIHC tumor samples (Fig. 4C) and corresponding decrease in *OCT4* and *SOX2* in *ZNF204P*-inhibited groups (Fig. 4D), we postulated that *ZNF204P* could function as a pluripotency regulator within cancer stem cells by decoying tumor suppressive miRNAs such as miRNA-145-5p. Collectively, our results show that *ZNF204P* is tumor-specific and exerts its oncogenic function through cell proliferation and migration, with possible function as competing endogenous RNA for tumor-suppressor miRNA-145-5p.

DISCUSSION

Outstanding issues despite the establishment of CSC model are i.) refining isolation and identification of CSCs through extracellular/intracellular factors and ii.) unraveling the complex mechanism of how such factors dysregulate self-renewal and

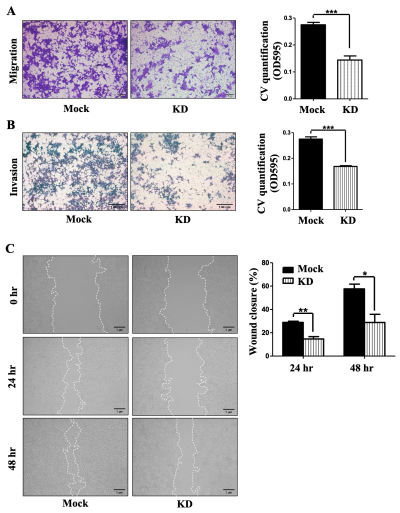

Fig. 3. *ZNF204P* affects cell migration and invasion. (A) Migration abilities of HepG2 cells slowed down by *ZNF204P* KD: Representative images (left) and crystal violet quantification (right). (B) Invasive properties of HepG2 cells similarly impeded by *ZNF204P* KD: Representative images (left) and crystal violet quantification (right). (C) Wound healing assay confirmed effect of *ZNF204P* on cell migration. Quantified wound closure (right) indicates that *ZNF204P* promotes cell movement. All data were acquired from three independent experiments (n = 2). *P < 0.05; **P < 0.01; ***P < 0.001 (two-tailed unpaired t-test analysis); ns indicates no significance.

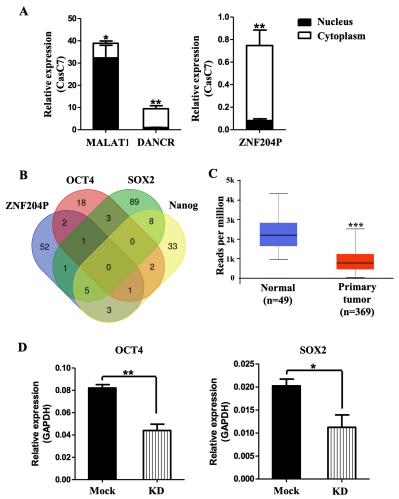

Fig. 4. Cytosolic *ZNF204P* as a miRNA-145-5p decoy, interfering with the latter's tumor suppressive role. (A) *ZNF204P* is located mostly in the cytoplasm relative to nucleus. (B) Predicted miRNA binding sites *ZNF204P*, *OCT4*, *SOX2* and *Nanog*. (C) Expression of tumor-suppressive miRNA-145-5p is down-regulated in tumor samples as visualized in UALCAN. (D) *ZNF204P* KD results in corresponding decrease in *OCT4* and *SOX2*. All data were acquired from three independent experiments (n = 2). *P < 0.05; **P < 0.01; ***P < 0.001 (two-tailed unpaired t-test analysis); ns indicates no significance.

extensive proliferation in CSCs. Extracellular markers such as *EpCAM*, *CD90* and *CD133* have successfully been used to isolate CSCs, while transcription factors such as *OCT4*, *SOX2* and *Nanog* are known tumorigenesis-driving players. Notably, such CSCs-specific markers are also highly functional in normal stem cells. Our study thus proposes a user-friendly method in identifying candidates through public data.

Here, we demonstrate the selection of stemness-related factors by identifying up-regulated genes in ESC and HCC samples respectively in microarray data via GEO2R. We reasoned that overlapping up-regulated genes from these two groups would shortlist factors conferring stemness properties by permitting continuous self-renewal and proliferative capabilities. Indeed, GO analysis of the shortlisted 139 factors displayed involvement in tumor-promoting signaling pathways and tumor cell growth. Moreover, previously characterized tumorigenesis-driving transcription factors such as *MYCN* and *MYB* were identified, indicating the robustness of our proposed method.

LncRNAs hold rich potential as therapeutic targets and prognostic markers as numerous studies indicate their contribution to self-renewal and proliferation of HCC CSCs – CUDR promotes malignant proliferation through multiple signaling pathways (22) while HOTAIR suppresses SETD2 in HCC CSCs (23). Thus, we selected the pseudogene-derived lncRNA *ZNF204P* for further investigation. To the best of our knowledge, our study is the first to publish experimental validation of *ZNF204P*, though it has been implicated as prognostic markers in various cancers (24) and a schizophrenia susceptibility allele (25). Given the complexities in perturbing lncRNAs (26), we opted to use CRISPRi/dCas9-KRAB system to block *ZNF204P* transcription, which entails proper annotation of transcription start sites (TSS). FANTOM CAT catalogues high-confidence TSSs through CAGE technique by capturing and single-molecule sequencing 5'ends of messenger RNAs (mRNAs) (27). Though with different transcription initiation evidence scores (TIEScore, Supplementary Table 2), we found that most of the *ZNF204P* transcripts corresponded to the same CAGE cluster, and thus designed sgRNAs +/− 200 relative to the defined TSS.

ZNF204P absence impaired cell survival while its knockdown reduced migratory/invasive properties. With ZNF204P up-regulated in TCGA-LIHC tumor samples, poor survival and enrichment of gene sets that drive cell proliferation and stem cell maintenance in ZNF204P-high group, we deduced that *ZNF204P* is a stemness-associated lncRNA with oncogenic capabilities. Furthermore, cytosolic *ZNF204P* shares a binding site with *OCT4* and *SOX2* for miRNA-145-5p, a well-studied tumor suppressor in cancer stem-like cells (20, 21). *ZNF204P* knockdown displays a corresponding decrease in *OCT4* and *SOX2*, suggesting a miRNA-145-5p/stemness-regulating transcription factor axis. We acknowledge that more in-depth studies are required to substantiate our stand, such as direct perturbation of miRNA-145-5p to examine its effect on HepG2 cells, but present our preliminary findings on *ZNF204P* as evidence of a simple yet comprehensive method to derive stemness-related factors.

In conclusion, we have identified a stemness-related factor *ZNF204P*, a pseudogene-derived lncRNA that modulates cell proliferation and migration as an oncogene, and possibly functions as a decoy of tumor-suppressive miRNA-145-5p by interfering with its degradation of *OCT4* and *SOX2* in HepG2 cells. With our findings, we propose that such oncogenic lncRNAs provide another avenue to interrogate gene circuits that control stemness within tumor mass, enabling selection of appropriate candidates as therapeutic targets.

MATERIALS AND METHODS

Data acquisition and analysis

GSE25097 microarray expression data was downloaded from HCCDB (28) to select HCC samples that displayed combinatorial elevated expression (> HCC average) of cancer stemness markers *EpCAM*, *Sall-4*, *Sox12* and *BMI-1* (Supplementary Table 1). GSE50206 (3 ESC/3 HDF samples) (29) and GSE25097 (7 HCC/6 Healthy samples) (30) microarray datasets were analyzed on GEO2R available at http://www.ncbi.nlm.nih.gov/geo/geo2r/ to derive differentially expressed genes. The following filters were used to list significantly dysregulated genes (Supplementary Table 1): P-value < 0.05, log2 FC > 3 (GSE50206), log2 FC > 0.5 (GSE25097). Resulting genes were overlapped to derive stemness-related candidates, and GO analysis was conducted on Enrichr (31).

Mean differential expression of selected genes (*ZNF204P*, *MYCN*, *MYB*) between normal and tumor sets were visualized using TCGA-LIHC data, and Kaplan-Meier survival plots were taken from UALCAN portal (32).

Processed RNA-seq V2 for LIHC (HCC) was downloaded from public TCGA data portal, and the dataset contains 422 samples (50 normal samples, 372 tumor samples). Fragments Per Kilobase of exon per Million (FPKM) reads were used.

TIE scores for genomic region spanning from chromosome 6:27324485 to chromosome 6:27440849 (*ZNF204P*) on FANTOM CAT were downloaded for quantification (Supplementary Table 2).

Association between miRNAs and target genes (*OCT4*, *SOX2*, *Nanog*, *ZNF79*, *ZNF204P*) were queried on miRSystem (33). Differential expression of resulting candidate miRNA-145-5p were visualized through UALCAN for TCGA-LIHC normal/tumor samples (49 normal, 369 tumor) (32).

Gene set enrichment analysis (GSEA)

GSEA software (version 4.1.0) was downloaded available at http://www.broadinstitute.org/gsea/downloads.jsp and expression data sets (.TXT format), phenotype labels (. CLS format) and gene sets (. GMX format) were created in accordance to GSEA specifications. Signature gene sets "Chiang Liver Cancer Subclass Proliferation Up" and "GOBP Regulation of Stem Cell Population Maintenance" were downloaded from Molecular Signatures Database (MSigDB v7.4) (34). Input for basic fields were: Enrichment statistic – weighted, Metric for ranking genes – Difference of class, Number of permutations – 1,000.

Cloning strategies for CRISPRi-mediated knockdown

sgRNAs targeting the regions encompassing transcription start site (+/− 200 relative to TSS) of *ZNF204P* was designed using CHOPCHOP v3 (35). The top and bottom strands of each targeting oligonucleotides were phosphorylated and annealed in thermocycler under settings: 37°C for 30 minutes, 95°C for 5 minutes, with ramping down to 25°C at a rate of 5°C/min. sgRNA sequences are included in Supplementary Table 1. Lenti_sgRNA_EFS_GFP (Addgene, #65656) vector (LRG) was digested with BsmBI (NEB) at 55°C for 1 hour, and subsequently annealed with each respective sgRNA duplex.

Cell culture and transfection

HCC cell-line HepG2 was maintained in DMEM (Invitrogen, USA) supplemented with fetal bovine serum 10% (Cytiva, SH30084.03), 1% penicillin-streptomycin (Gibco, #15140122) in 37°C in 5% CO2 humidified incubator. Cells were passaged every 2-3 days and harvested using 0.25% Trypsin-EDTA (Invitrogen, #25200-056). For knockdown studies, 1×10^5 cells were seeded per well in a 24-well plate one day prior to transfection. sgRNA-encoding LRG (600 ng) and modified dCas9-KRAB-MeCP2 (300 ng) were mixed with 2 µl of Lipofectamine 3000 (Thermo Fisher Scientific, # L3000015) and incubated at room temperature for 5 minutes. Transfection mixture was added to each well, and cells were harvested for downstream analysis at 24th and 48th hour time-points.

Cell viability assay

100 µl of cell media from each well (duplicates) in 24-well plate was collected and transferred to a 96-well plate at the 24th and 48th hour post-perturbation. 10 µl of CCK-8 solution (Dojindo, CK04-13) was added to each well, and incubated for 2 hours at 37°C, 5% CO2. The absorbance was read at 450 nm using TECAN microplate reader (Infinite 200 PRO), and the average value of duplicates were taken for reading of each group.

Migration and invasion assays

100 µl of cell suspension (DMEM-1% penicillin/streptomycin with no serum) containing 5×10^3 cells subjected to CRISPRi for 24 hours were dispensed into the Transwell insert (Corning, 3422) and incubated for 10 minutes at 37°C and 5% CO2. 600 µl of media solution (DMEM-1% penicillin/streptomycin with fetal bovine serum) was added to the bottom of lower chamber. 48 hours post-incubation, inserts were removed from plate for further analysis. For image taking, remaining cells from the membrane top were wiped off using a cotton-tipped applicator. Inserts were subsequently fixed in ice-cold 100% methanol for 15 minutes, washed in PBS and stained with 1% crystal violet solution (Sigma Aldrich) for 30 minutes at room temperature. After rinsing in water, inserts were left to dry, and images were taken at 10× magnification using Olympus IX51 light microscope. For crystal violet quantification, cut-out insert membranes were solubilized in 250 µl of 1% sodium dodecyl sulfate solution for 10 minutes at room temperature. 50 µl (in triplicates) were transferred to 96-well plate for OD reading at 595 nm.

The same steps were repeated for invasion assays, with the exception of using inserts pre-coated with Matrigel (Corning, 354234) at diluted concentration of 200 ug/ml.

Wound-healing assay

2×10^5 cells were seeded per well in 24-well plates pre-coated with 0.01% Poly-L-lysine (Sigma, P2636-25MG) one day prior to transfection. '0 hour' time-point was established at 6 hours post-transfection, and each wound was made using a 200 ul pipette tip. Images were taken at 10x magnification using Olympus IX51 light microscope at stated time-points. Wound area was quantified through ImageJ available at https://imagej.nih.gov/ij/download.html using the polygon selection mode.

Colony formation assay

Transfected 2×10^3 cells per well were seeded in 6-well plates, and incubated for 10-14 days. All groups underwent PBS wash, 30 minute fixation with ice-cold 100% methanol, staining (0.5% crystal violet solution) for 2 hours at room temperature, and air-drying for at least 2 hours following washes with tap water. Images were taken at 10x magnification using Olympus IX51 light microscope at stated time points.

RNA isolation and quantitative real-time PCR

0.5-1 µg of RNA was used in synthesis of 1st strand complementary DNA (cDNA) using Superscript II reverse transcriptase (Thermo Fisher Scientific, #18064014) and oligo (dT) primers (Macrogen). The resulting cDNA was diluted ×10 in nuclease-free water and stored at −20°C. Quantitative PCR (qPCR) was carried out in triplicates using KAPA SYBR®FAST Bio-Rad Icycler 2X Qpcr Master Mix (Roche, KK4608). For RNA fractionation, 2×10^6 HepG2 cells were harvested for fractionated RNA isolation using PARIS kit (Ambion, AM1921), as per manufacturer's protocol. All qPCR primer sequences are included in Supplementary Table 3.

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AUTHOR CONTRIBUTIONS

J-H.H and J.L performed the experiments. JW.L and K.H.B.C analyzed the data. W.Y.C and M.J.K contributed to data interpretation and manuscript writing. J-H.H, L.K.K and Y-J.K conceived the study and wrote the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors have no conflicting interests.

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