SHARPIN promotes cell proliferation of cholangiocarcinoma and inhibits ferroptosis via p53/SLC7A11/GPX4 signaling

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Abstract
SHARPIN is a tumor-associated gene involved in the growth and proliferation of many tumor types. A function of SHARPIN in cholangiocarcinoma (CCA) is so far unclear. Here, we studied the role and function of SHARPIN in CCA and revealed its relevant molecular mechanism. The expression of SHARPIN was analyzed in cholangiocarcinoma tissues from patients using immunohistochemistry, quantitative PCR, and western blot analysis. Expression of SHARPIN was suppressed/overexpressed by siRNA silencing or lentiviral overexpression vector, and the effect on cell proliferation was determined by the CCK-8 assay and flow cytometry. Accumulation of reactive oxygen species was measured with MitoTracker, and JC-1 staining showed mitochondrial fission/fusion and mitochondrial membrane potential changes as a result of the silencing or overexpression. The ferroptosis marker solute carrier family 7 member 11 (SLC7A11), glutathione peroxidase 4 (GPX4), and the antioxidant enzymes superoxide dismutase 1 (SOD-1) and SOD-2 were analyzed by western blot. The results showed that SHARPIN expression was increased in CCA tissue, and this was involved in cell proliferation. SHARPIN silencing resulted in accumulated reactive oxygen species, reduced mitochondrial fission, and a reduced mitochondrial membrane potential. Silencing of SHARPIN inhibited the ubiquitination and degradation of p53, and downregulated levels of SLC7A11, GPX4, SOD-1, and SOD-2, all of which contributed to excessive oxidative stress that leads to ferroptosis. Overexpression of SHARPIN would reverse the above process. The collected data suggest that in CCA, SHARPIN-mediated cell ferroptosis via the p53/SLC7A11/GPX4 signaling pathway is inhibited. Targeting SHARPIN might be a promising approach for the treatment of CCA.

Keywords
cholangiocarcinoma, ferroptosis, mitochondria, proliferation, SHARPIN
1 | INTRODUCTION

Cholangiocarcinoma (CCA) is a rare malignant adenocarcinoma that arises from the epithelial cells of the intra- and extra-hepatic bile ducts. It is the second most common hepatobiliary malignancy after hepatocellular carcinoma. The incidence and mortality of CCA has increased worldwide over the past 30 years, which may be attributed to the early and strong aggressiveness of CCA. The diagnosis of cholangiocarcinoma is very difficult because in the early stages, the disease is asymptomatic. Patients are often diagnosed in the later stages of disease, missing the window of opportunity for optimal treatment. Chemotherapy, radiotherapy, and surgical resection are the main approaches for treatment of CCA. However, these treatments do not achieve satisfactory results. Research to disclose the mechanism of cholangiocarcinogenesis and its progression, as well as the identification of new makers, is urgently needed to improve therapeutic measures of CCA.

Shank-associated RH domain interacting protein (SHARPIN) is a component of the linear ubiquitin chain activation complex. Previous studies have recognized SHARPIN as a novel modulator of immune and inflammatory responses. Recently, SHARPIN was identified as a tumor-associated gene involved in inhibition of apoptosis, mediation of tumor progression, enhancement of cell detachment and migration, and activation of survival signaling pathways. In addition, SHARPIN was found to mediate the cell fate through the action of mitochondria, although the detailed mechanism for this is not fully elucidated. We considered the possibility that SHARPIN might regulate cell ferroptosis via mitochondria and that this might be relevant to CAA. To test this hypothesis, we investigated the effects of SHARPIN in mediating CAA cells to enter ferroptosis by alteration of mitochondrial function.

Ferroptosis is a recently recognized type of regulated cell death that differs from other types of regulated cell death in morphology, genetics, and biochemistry. Mitochondria are involved in ferroptosis, and cysteine deprivation also plays a crucial role. Recent studies have shown that ferroptosis is involved in several diseases, and it has become the focus of research on the treatment and prognosis of cancer. Mechanistically, ferroptosis is triggered by suppressing a central regulator, glutathione peroxidase 4 (GPX4), which is required for the clearance of reactive oxygen species (ROS). Reduced GPX4 activity can result from direct inhibition of its enzyme activity or promoted degradation by inducers. Indirect inhibition of GPX4 can also occur through blocking a glutamate/cysteine antiporter that exports intracellular glutamate in exchange for extracellular cysteine to generate glutathione. Inhibition of cysteine uptake by p53 can sensitize cells to ferroptosis through downregulating the expression of SLC7A11 and GPX4, reduction of antioxidant capacity and resultant ROS accumulation. In addition, SHARPIN has been reported to facilitate p53 poly-ubiquitination and its degradation in breast cancer.

Whether SHARPIN functions in CCA was previously unclear, but here we confirm that SHARPIN expression is increased in CCA, promotes cell proliferation, and suppresses ferroptosis. The present study identified the mechanism by which SHARPIN promotes CCA proliferation via the p53/SLC7A11/GPX4 signaling pathway.

2 | MATERIALS AND METHODS

2.1 | Ethics, consent, and permissions

All experiments with human samples were approved by the Ethical Committee of Medical Research, Shunde Hospital, Southern Medical University (The First People's Hospital of Shunde, China).

2.2 | Cell culture and clinical tumor tissue

The two human CCA cell lines, HuCCT1 and Hccc9810, were purchased from the American Type Culture Collection. Ferrostatin-1(#HY-100579) was purchased from MCE. Cells were maintained in exponential growth phase in RPMI-1640 medium (Life Technologies) containing 10% FBS (Invitrogen), 100 U/mL penicillin, and 0.1% (w/v) streptomycin at 37°C in a humidified atmosphere of 5% CO2. Cells were used within 6 months of resuscitation. CCA tissue samples were obtained by surgical resection. The tumor and adjacent tissues extracted from clinical cases were formalin-fixed, paraffin-embedded, and sectioned for immunohistochemistry and immunofluorescence assays or immediately frozen for western blot and RT-qPCR assays.

2.3 | Establishment of SHARPIN silenced and stable overexpression cell lines

The HuCCT1 and Hccc9810 cells were transfected using Lipofectamine 3000 reagent (Invitrogen) for 72h according to the manufacturer’s instructions. The SHARPIN siRNA (target sequence 5'-CCCT GAGTGTTCAGCTTCA-3') and negative-control siRNA (scramble siRNA) (target sequence 5'-GGCTTAGAAAGCCTATGC-3') were transfected into cells with 5 μg siRNA, at a ratio of 1:1. Lentiviral vectors (SHARPIN EXP) and control (SHARPIN EXP NC) (Genomeditech, Shanghai, China) for SHARPIN overexpression (MOI = 10) were used with polybrene to transfect HuCCT1 and Hccc9810 cells; 48h later, the medium was replaced with antibiotics to acquire single cell cloning. The silenced and overexpression cell lines were confirmed by western blot and RT-qPCR assay. To explore the effect of SHARPIN in ferroptosis, the SHARPIN silenced cell lines (HuCCT1 and Hccc9810 cells) were treated with or without a ferroptosis inhibitor Ferrostatin-1 (1 μM), and the ferroptosis hallmark GPX4 and SLC7A11 were detected.

2.4 | Cell viability and proliferation assay

After transfection, the viability of the transfected SHARPIN siRNA and lentiviral vectors cells were determined using the CCK8 assay. Cells were plated in 96-well plates with 5 × 10^3 cells per well in three replicate wells. CCK-8 reagent (Dojindo Molecular Technologies) was added to each well (10 μL) and incubated for 1h. Absorbance at 450nm was recorded using a microplate reader (Victor Nivo 3F, Perkin Elmer). For the EdU proliferation assay, cells were cultured, fixed, blocked, and incubated with EdU dyes (Thermo Fisher Scientific). The nuclei were stained with DAPI.
stained with Hoechst dye 33,342 (Solarbio, life sciences) for 30 min at room temperature. Images of five randomly selected areas were recorded with a fluorescence microscope (Leica, DMi8). For flow cytometry, cell proliferation was analyzed without Hoechst dye 33,342 staining. Data were acquired using a flow cytometer (FACSAria, Becton Dickinson) and analyzed with FCS Expression software (3.0).

### 2.5 Reactive oxygen species production assay

The cells were seeded in plates at 2 × 10^5 cells per well and cultured overnight, then incubated with the ROS-detecting fluorescence dye 2,7-dichlorofluorescein diacetate (DCF-DA, 10 μM) at 37°C for 30 min. Partial cells were washed with PBS, intracellular ROS content was measured by flow cytometry, and partial cells were microscopically examined. Data were analyzed with FCS Expression software and Image J software.

### 2.6 Mitochondrial membrane potential assay

The mitochondrial membrane potential was quantified by Mito Tracker Red staining (Beyotime). Cells were incubated in Mito Tracker Red solution (final concentration, 30 nM) for 30 min. The cells were washed three times with PBS, and incubated with DAPI solution for 5 min in the dark. The cells were visualized under an inverted fluorescence microscope (Zeiss). In addition, the MitoProbe JC-1 Assay Kit (Thermo Fisher Scientific Inc) was used to detect changes in mitochondrial membrane potential. The results of the latter assay were obtained by flow cytometry.

### 2.7 RNA extraction, cDNA synthesis, and real-time RT-PCR

Gene expression was determined by qRT-PCR as previously described. Total RNA was isolated from cells or tissue samples using Trizol (Invitrogen). The following primers were used: SHARPIN sense 5'-TGTTCCTCAGCTCGGTCTT-3' and antisense 5'-AGTTCCGTCCTCATCTT-3', β-actin sense 5'-AACAGTCCGCTAGACAC-3' and antisense 5'-CGTTGACA TCCGTAAGACC-3'. Fluorescence was detected using a CFX96 Touch instrument (Bio-Rad). Each sample was run in triplicate and compared with β-actin as the reference gene. Results were analyzed using the 2^-ΔΔCT method for the relative quantification of mRNA expression.

### 2.8 Western blot analysis

Western blot analysis was performed as previously described. Briefly, total protein was isolated using RIPA lysis buffer with PMSF. Protein concentration was determined using the bicinchoninic acid assay. Following 12% SDS-PAGE electrophoresis, proteins were transferred to PVDF membranes that were blocked with 5% BSA at room temperature for 1 h. The blots were probed with anti-SHARPIN (1:4000, # ab197853, Abcam), anti-actin (1:1000, #AA128, Beyotime), anti-GPX4 (1:1000, # ab125066, Abcam), anti-SLC7A11 (1:500, # 26864-1-AP, Proteinch), anti-p53 (1000, # ab26, Abcam), anti-SOD-1 (1:1000, # ab51254, Abcam), anti-SOD-2 (1:1000, # ab68155, Abcam), and anti-AIFM2/FSP1 (1:1000, # 20886-1-AP, Proteintech) followed by the appropriate secondary antibody, which was conjugated to HRP (anti-mouse IgG/anti-rabbit IgG, CST, 1:5000). Bands were visualized by chemiluminescence (#35050, Thermo Fisher) and quantified by ChemiDoc Touch (Bio-Rad). Data were analyzed with Quantity One analysis software (Bio-Rad).

### 2.9 Immunohistochemistry

The tumor specimens were fixed in 4% formalin and embedded in paraffin. Sections were cut into 5 μm slices, antigen retrieval was performed with citrate buffer, and endogenous peroxidase activity was blocked with 3% H₂O₂. Samples were incubated with anti-SHARPIN antibodies (1:4000, # ab197853, Abcam) overnight at 4°C. After washing with PBS, the slices were incubated with HRP-conjugated secondary antibodies at 37°C for 30 min. Signals were detected with DAB. The nuclei were counterstained with hematoxylin for 1 min, washed three times with PBS, and examined by microscopy. The positively stained area was quantified using Image J software.

### 2.10 Immunofluorescence

Tissue slices were obtained as described above. Slices were treated as described previously. DAPI was used to stain cell nuclei. Immunostaining was then examined using a Leica microscope (DM4B). Cells were analyzed with image- Pro Plus 6.0 (IPP 6.0; Media Cybernetics).

### 2.11 Statistical analysis

Statistical analysis of data was performed using GraphPad Prism 6.0 (GraphPad Software) as previously described. All data are presented as mean ± SD from triplicate experiments and statistical significance is shown as *p < 0.05, **p < 0.01, and ***p < 0.001.

### 3 RESULTS

#### 3.1 Expression of SHARPIN is elevated in cholangiocarcinoma

To investigate the role of SHARPIN in cholangiocarcinoma, we first analyzed SHARPIN expression in CCA cancer specimens and in normal controls based on the publicly available database Gene Expression Profiling Interactive Analysis (GEPIA). This revealed...
(A) The mRNA expression of Sharpin

Normal (n = 9) Tumor (n = 36)

(B) Adjacent Tumor

(C) SHARPIN DAPI Merge

Adjacent Tumor

(D) Mean fluorescence intensity of SHARPIN

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(E) The mRNA expression of SHARPIN

Adjacent (n = 8) Tumor (n = 7)

(F) SHARPIN β-ACTIN

40 kDa 43 kDa

(A) T A T A T

(G) The expression of SHARPIN

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Adjacent Tumor
that SHARPIN mRNA levels were elevated in cholangiocarcinoma tissue samples compared to normal tissue (Figure 1A). The association between SHARPIN expression and prognosis of CCA patients through the publicly available database GEPIA was also shown (Figure S1). This finding was confirmed by detection of SHARPIN expression by immunohistochemistry and immunofluorescence in paired samples from patients with CCA (Figure 1B–D). Both qPCR and western blot results showed elevated SHARPIN expression in tumor tissue compared to adjacent healthy tissue (Figure 1E–G). These findings suggested that SHARPIN expression was increased in CCA.

3.2 | SHARPIN promotes growth of cholangiocarcinoma cells

Two CCA cell lines (Hccc9810 and HuCCT1) were transfected with SHARPIN siRNA and lentiviral overexpression vectors. The knockdown efficiency was confirmed by western blot and qPCR (Figure S2). The CCK-8 assay was performed to demonstrate that siSHARPIN had significantly decreased CCA cell viability and overexpression of SHARPIN would increase CCA cell viability (Figure 2A,B). The relationship between cell proliferation and SHARPIN expression was evaluated using the EdU cell proliferation assay combined with flow cytometry and fluorescence microscopy. Consistent with the findings on cell viability, the CCA cells transfected with siSHARPIN were significantly inhibited in their proliferation, and overexpression of SHARPIN would increase CCA cell proliferation (Figure 2C–F). Collectively, these results showed that SHARPIN played a critical role in promoting CCA cell proliferation.

3.3 | Silencing SHARPIN induced cholangiocarcinoma cells to generate reactive oxygen species in vitro

The effect of SHARPIN expression on cell ROS production was evaluated. Intracellular ROS levels were measured using the ROS-detecting fluorescence dye DCF-DA Fluorescent microscopy. The result showed that the ROS levels in siSHARPIN-transfected cells were significantly increased compared to scramble siRNA, and SHARPIN EXP would significantly reduce the level of ROS compared with SHARPIN EXP NC (Figure 3A). This was confirmed by quantitative analysis (Figure 3B). Consistent with the fluorescent results, flow cytometry detected the effect of knockdown and overexpression SHARPIN on ROS generation. The ratio of DCF-DA-positive cells in siSHARPIN-transfected cells was significantly higher than
the scramble siRNA, and SHARPIN EXP was significantly lower than the SHARPIN EXP NC (Figure 3C,D). Thus, these results suggest that SHARPIN is involved in ROS generating in CCA cells.

3.4 | Knockdown SHARPIN inhibits the function of mitochondria

Previous studies have shown that excessive ROS generation can alter the shape of mitochondria, and this contributes to cell apoptosis. The role of SHARPIN in mitochondria morphology was studied by an immunocytochemistry assay (see Methods). The siSHARPIN-transfected CCA cells displayed reduced abundance of mitochondrial membranes and fission compared with the control, and SHARPIN EXP was significantly higher than the SHARPIN EXP NC (Figure 4A,B). Whether changed SHARPIN expression influenced the mitochondrial membrane potential ($\Delta \psi_m$) was assessed using the fluorescent dye JC-1, which introduces a fluorescence shift from red to green if the mitochondrial membrane potential decreases. This shift was quantified by flow cytometry for evaluation of mitochondrial involvement. The fraction of cells displaying green fluorescence was increased in siSHARPIN-transfected CCA cell lines compared to scramble siRNA, and SHARPIN EXP had more red fluorescence compared with SHARPIN EXP NC (Figure 4C,D). This suggests that changes in SHARPIN expression alter the mitochondrial membrane potential.

3.5 | SHARPIN inhibits cellular ferroptosis through a p53/SLC7A11/GPX4 cascade

To further determine the molecular mechanism by which SHARPIN regulates ROS production and inhibits cell proliferation, western blots were performed that were stained for SHARPIN, p53, GPX4, solute carrier family 7 member 11 (SLC7A11), apoptosis-inducing factor mitochondria-associated 2 (AIFM2, also called ferroptosis suppressor
protein 1 (FSP1)), superoxide dismutase 1 (SOD-1), and superoxide dismutase 2 (SOD-2). The results show that knockdown of SHARPIN expression increased the expression of p53, and overexpression of SHARPIN would not change the level of p53. However, the ferroptosis biomarker proteins SLC7A11 and GPX4 were reduced. In addition, the antioxidant proteins SOD-2 (cytosolic) and SOD-1 (mitochondrial) were both downregulated following SHARPIN silencing (Figure 5A–H). Overexpression of SHARPIN in CCA cell lines would increase the SLC7A11, GPX4, SOD-2, and SOD-1. In addition, neither knockdown nor overexpression SHARPIN altered the level of FSP1 (Figure 5A–H). Furthermore, we employed co-immunoprecipitation to assay the protein interaction between p53 and ubiquitin. The data showed that knockdown SHARPIN reduced physical interactions between p53 and ubiquitin (Figure 5I). These observations suggest that SHARPIN inhibited ubiquitination and degradation of p53 via the SLC7A11/GPX4 signaling pathway. To further confirm the role of SHARPIN in mediating cell ferroptosis, the cell lines were transfected with SHARPIN siRNA and treatment with or without the ferroptosis inhibitor ferrostatin-1. The ferroptosis hallmark SLC7A11 and GPX4 had no obvious change compared with scramble (Figure S3). In this way, SHARPIN would protect cells from ferroptosis and promote cell proliferation. This is summarized in Figure 6.

4 | DISCUSSION

Cholangiocarcinoma is a highly aggressive liver tumor with high mortality rates that can be molecularly heterogeneous, and its incidence is increasing. In China, the incidence of CCA currently increases by more than 5% per year. In the search for factors that
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Using immunofluorescence, immunohistochemistry and molecular assays (western blots and qPCR), we demonstrate that SHARPIN expression is significantly increased in tumor tissue and that its silencing/overexpression results in a reduced/promoted ability for cell growth and proliferation. This finding is consistent with previously reported roles of SHARPIN in multiple tumor studies.

Our work contributes to a growing body of literature that identifies SHARPIN as a potential target for tumor therapy, in this case to combat CCA. Inhibition of SHARPIN expression enhances intracellular ROS production and reduces mitochondrial fission, together with a reduction in mitochondrial membrane potential. Overexpression of SHARPIN reduces intracellular ROS and protects the function of mitochondria. Mitochondria was in dynamic flux of fusion and fission, and this process depends on gene expression and the intracellular microenvironment. It was shown that enhanced mitochondrial fission could promote cancer growth. This would contribute to repressing respiratory complexes I and IV, while the respiratory complexes I and III, which were the main sources of ROS, would remain unaffected. That way, repression of complex I by fission would induce the production of mitochondrial ROS and subsequently raise intracellular ROS levels. This would lead to impaired mitochondrial membrane potential. These findings obtained with JC-1 staining are in line with this, as they revealed that silencing SHARPIN promotes the JC-1 red to green shift, and overexpression of SHARPIN will reverse this process. Thus, it is most likely that SHARPIN mediates cell growth and proliferation through changing the function of mitochondria.

At the molecular level, we found that reducing SHARPIN expression increased the level of p53 protein. A pervious study demonstrated that in human breast cancer, SHARPIN could facilitate p53 poly-ubiquitination and degradation in an MDM2-dependent manner. By application of co-immunoprecipitation, we were able to show that interactions of SHARPIN and p53 were mediated by SHARPIN via the ubiquitin-dependent pathway in

FIGURE 5 Changing the expression of SHARPIN would determine cell fate with ferroptosis. (A) Western blots showing expression of p53, SHARPIN, FSP1, glutathione peroxidase 4 (GPX4), solute carrier family 7 member 11 (SLC7A11), superoxide dismutase 1 (SOD-1), and superoxide dismutase 2 (SOD-2). β-actin served as an internal reference. (B–H) Quantitative analysis of p53, SHARPIN, FSP1, GPX4, SLC7A11, SOD-1, and SOD-2 based on band density (I) Co-immunoprecipitation assay of ubiquitin bound to p53 protein in the Hucc9810 CCA cell line following SHARPIN silencing. Values (mean ± SD) from quintuplicate experiments are shown. *p < 0.05, **p < 0.01 vs Scr siRNA group, #p < 0.05 vs SHARPIN NC group.
human CCA cells. We also noticed that the cell ferroptosis markers SLC7A11 and GPX4 were significantly reduced, as were the antioxidant enzymes SOD-1 and SOD-2. SLC7A11 is a plasma membrane cysteine/glutamate antiporter. Inhibition of SLC7A11 contributes to ferroptotic cell death. The gene for GPX4 is located downstream of SLC7A11, which functions as a guard against ferroptosis induced by lipid ROS. Overexpression of GPX4 would thus block ferroptosis, while depletion or repression of its expression would augment the levels of ROS. Cysteine uptake is inhibited by p53, and this sensitizes the cells to ferroptosis by repression of SLC7A11 and promotion of ROS production. A later study revealed that the p53/SLC7A11/GPX4 signaling pathway is involved in mediating ferroptosis in prostate cancer cells. We also found that SLC7A11 and GPX4 had not obviously changed after SHARPIN siRNA transfected CCA cell line treatment with ferroptosis inhibitor. Furthermore, we found SHARPIN was not able to alter the expression of FSP1, which was a maker of the FSP1-CoQ10 (GSH/GPX4-independent) ferroptosis pathway. These results suggest that SHARPIN mediated CCA cells ferroptosis through the GSH/GPX4-dependent rather than the FSP1-CoQ10 pathway. ROS production can impair mitochondrial membrane potential. The cytoplasmic enzyme SOD-1 and the mitochondrial SOD-2 are important antioxidant enzymes. Both SOD-1 and SOD-2 are responsible for protecting the cell from oxidant stress, and our data show that SHARPIN silencing contributes to reduced SOD-1 and SOD-2 activity, thus promoting cell ferroptosis in CCA.

Here we demonstrate that SHARPIN is overexpressed in CCA, which inhibits ferroptosis and promotes cell proliferation. Our results further reveal that silencing of SHARPIN reduces p53 ubiquitination and, as a consequence, the expression of SLC7A11 and GPX4 is decreased, resulting in elevated levels of intracellular and mitochondrial oxidative stress, which will eventually lead to ferroptosis. Taken together, these findings uncover a SHARPIN/p53/SLC7A11/GPX4 axis in CCA cells, which may facilitate the targeting of SHARPIN as a novel therapeutic strategy for treatment of human CCA.

**AUTHOR CONTRIBUTIONS**

J.Y. and W.W. designed the research, and B.Q. analyzed the data. J.Y. and C.Z. wrote the paper; C.Z., J.L, and K.Z. performed the experiments; H.O., B. W., and X. Z. purchased the reagents and materials; L.Z., K.S, and X.D. provided guidance on experimental technology. All authors read and approved the final manuscript.

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**DISCLOSURE**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
DATA AVAILABILITY STATEMENT
The data supporting the findings of this study are available from the corresponding author upon reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
Approval of the research protocol by an Institutional Reviewer Board: The study protocol was approved by the Investigation Ethical Committee of Shunde Hospital of Southern Medical University. Informed Consent: All patients or their guardians were given and accepted informed consent. Registry and the Registration No. of the study/trial: The study protocol was approved by the Investigation Ethical Committee of Shunde Hospital of Southern Medical University (registration no. 20200101).

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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