


ORIGINAL ARTICLE

Regulation of XPO5 phosphorylation by PP2A in hepatocellular carcinoma

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Abstract

Exportin 5 (XPO5) is a shuttle protein that mediates precursor miRNA (pre-miRNA) export from the nucleus to the cytoplasm, an important step in miRNA maturation. We previously demonstrated that XPO5 was phosphorylated by ERK kinase and subsequently underwent conformation change by the peptidyl-prolyl isomerase Pin1, leading to the reduced miRNA expression in hepatocellular carcinoma (HCC). Protein phosphorylation modification serves as a reversible regulatory mechanism precisely governed by protein kinases and phosphatases. Here we identified that the phosphatase PP2A catalyzed XPO5 dephosphorylation. PP2A holoenzyme is a ternary complex composed of a catalytic subunit, a scaffold subunit, and a regulatory subunit that determines substrate specificity. In this study, we characterized the involvement of B55 β subunit in XPO5 dephosphorylation that favored the distribution of XPO5 into the cytoplasm and promoted miRNA expression, leading to HCC inhibition in vitro and in vivo. Our study demonstrates the regulatory role of B55 β -containing PP2A in miRNA expression and may shed light on HCC pathogenesis.

KEYWORDS

hepatocellular carcinoma, microRNA, PP2A, XPO5

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies with high lethality and recurrence worldwide.¹ The onset and progression of HCC involve multiple external factors combined with altered expression of proteins and noncoding RNAs (ncRNAs). MicroRNAs (miRNAs) represent small ncRNAs of 20–22 nucleotides that tunes gene expression at post-transcriptional level

to maintain homeostasis.² However, the overall expression of miRNAs is downregulated in HCC, which is largely attributed to the defective miRNA biogenesis.^{3–5} Therefore, clarifying the molecular mechanisms underlying miRNA biogenesis is essential for understanding HCC pathogenesis.

The canonical miRNA biogenesis initiates with the transcription of miRNA genes mainly by RNA polymerase II into primary transcripts (pri-miRNAs), which can be

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subsequently spliced by RNase III Drosha to produce precursor miRNAs (pre-miRNAs) with a stem loop structure.⁶ With the assistance of RanGTP, exportin 5 (XPO5) transports pre-miRNAs into the cytoplasm, where they further undergo cleavage by another RNase III Dicer and are loaded onto argonaute (AGO) protein to generate the effector complex called RNA-induced silencing complex (RISC).⁷

XPO5-mediated export of pre-miRNAs is an important step for miRNA maturation.^{8–11} Overexpression of XPO5 enhances export efficiency and miRNA function.⁹ During cell cycle entry, XPO5 can be promptly induced and serves as a critical molecular hub controlling gene expression via a global elevation of miRNAs.¹² However, the dysregulation of XPO5 occurs in cancer, which yields profound effect on miRNA expression and aggravates tumorigenesis.^{13,14} In a subset of tumors with microsatellite instability, a genetic defect in XPO5 traps pre-miRNAs in the nucleus and reduces miRNA processing, whereas reexpression of the wild-type XPO5 reverses the impaired pre-miRNA export and the aggressive cellular phenotype, indicating XPO5's tumor-suppressive property.¹³ Our previous studies showed that ERK kinase-mediated phosphorylation of XPO5 at proline-directed serine/threonine sites, coupled with the peptidyl-prolyl isomerase Pin1-catalyzed conformation change, impaired the nuclear export of pre-miRNAs and downregulated miRNA expression during HCC development,^{15–18} highlighting the important role of phosphorylation in determining XPO5 function. Phosphorylation is a reversible regulatory mechanism precisely controlled by protein kinases and protein phosphatases. Given that serine/threonine phosphatase PP2A can coordinate with Pin1 and catalyze the dephosphorylation of proline-directed serine/threonine sites on many proteins, such as c-Myc, Cdc25c, and Tau,^{19,20} we hypothesized that PP2A could be involved in the dephosphorylation of XPO5.

PP2A belongs to the phosphoprotein phosphatase (PPP) superfamily and maintains cellular homeostasis by counteracting many kinase-driven signals.²¹ The core enzyme of PP2A is composed of a scaffold subunit (A subunit) and a catalytic subunit (C subunit). To achieve full activity toward particular substrates, the core enzyme further interacts with a variable regulatory subunit (B subunit) to constitute the heterotrimeric PP2A holoenzyme.^{22,23} The B subunit can fall into four unrelated families: B (B55), B' (B56), B'' (B72, B130, PR48, and G5PR), and B''' (Striatin).²⁴ The structure of these subunits varies greatly, allowing for the association of PP2A with diverse substrates. For example, the alpha isoform of B55 is responsible for the dephosphorylation of threonine 308 of Akt,²⁵ while the alpha isoform of B56 interacts with c-Myc and regulates the phosphorylation status of serine 62.¹⁹ In mammals, both A and C subunits are ubiquitously expressed, while the abun-

dance and subcellular localization of B subunits are tissue and developmental-stage specific.²⁶ Given that distinct B subunits associate with the overlapping binding sites on the A subunit within PP2A core enzyme in a mutually exclusive manner,²⁷ the B subunit dictates subcellular compartmentalization and substrate specificity for PP2A.

In this study, we identified that the beta isoform of B55 (B55 β)-containing PP2A is involved in the dephosphorylation of XPO5, thus facilitating XPO5-mediated miRNA maturation and HCC suppression. Moreover, the expression of B55 β was downregulated in HCC tumor samples. Therefore, our findings demonstrate the regulatory role of B55 β -containing PP2A in miRNA expression and HCC pathogenesis, suggesting a novel target for HCC therapy.

2 | RESULTS

2.1 | PP2A is responsible for the dephosphorylation of XPO5

The PPP family members share high homology in the catalytic subunits.^{21,23} In order to determine which phosphatase is involved in the dephosphorylation of XPO5, we overexpressed constitutively active MEK (MEKDD) in HEK-293T cells to activate ERK/XPO5 cascade and subsequently transfected these cells with distinct catalytic subunits of serine/threonine phosphatases (PP1, PP2B, and PP2A). The level of phosphorylated XPO5 (p-XPO5) was decreased upon the overexpression of the C subunit of PP2A (Figure 1A). To further validate the effect of PP2A on XPO5, we transfected HEK-293T cells with plasmids expressing myc-tagged XPO5, and immunoprecipitated XPO5 protein with antibody to the myc tag. Clearly, the C subunit of PP2A downregulates p-XPO5 level (Figure 1B). To test whether XPO5 protein could complex with the PP2A core enzyme, co-immunoprecipitation was performed and the protein complexes were analyzed. As shown in Figure 1C, XPO5 was specifically recovered with the C subunit of PP2A. These results indicate that PP2A participates in XPO5 dephosphorylation.

2.2 | The PP2A regulatory subunit B55 β mediates the dephosphorylation of XPO5

Considering that the B subunit determines the specificity of PP2A substrate, we next tended to identify the B subunit specific for XPO5 dephosphorylation. HEK-293T cells were transfected with the available B subunits in hand and p-XPO5 level was measured. As indicated in Figure 2A, B55 β subunit particularly decreased p-XPO5, suggesting the involvement of B55 β in XPO5 dephosphorylation. For

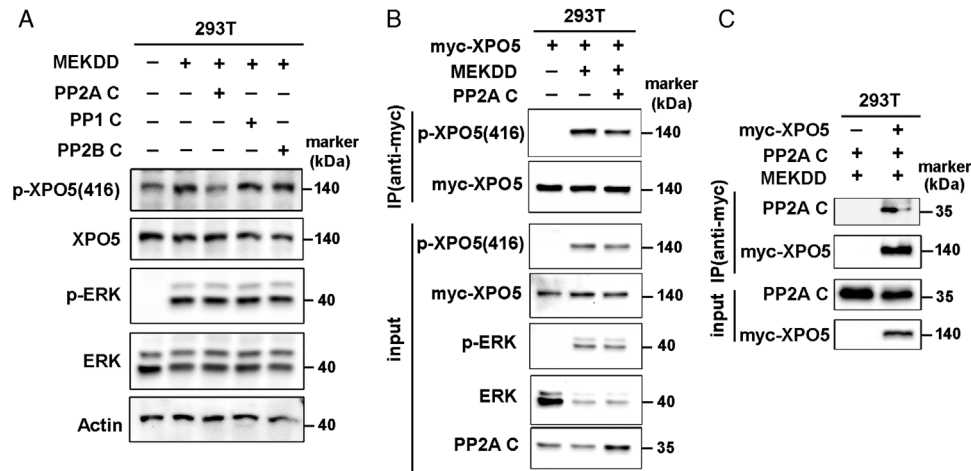


FIGURE 1 PP2A catalyzes the dephosphorylation of XPO5. (A) HEK-293T cells were transfected with plasmids expressing MEKDD and different catalytic subunits of serine/threonine phosphatases (PP1, PP2B, and PP2A). The phosphorylated and total XPO5/ERK proteins were detected via Western blot. (B) HEK-293T cells were transfected with plasmids expressing MEKDD, myc-XPO5 and C subunits of PP2A and myc-XPO5 was immunoprecipitated from cell lysates. The immunoprecipitates were immunoblotted with the indicated antibodies. (C) The lysates from HEK-293T cells co-transfected with the indicated plasmids were subjected to immunoprecipitation via anti-myc antibody. The enriched complexes were immunoblotted with the indicated antibodies. MEKDD: constitutively active MEK; p-XPO5 (416): phosphorylated XPO5 at serine 416.

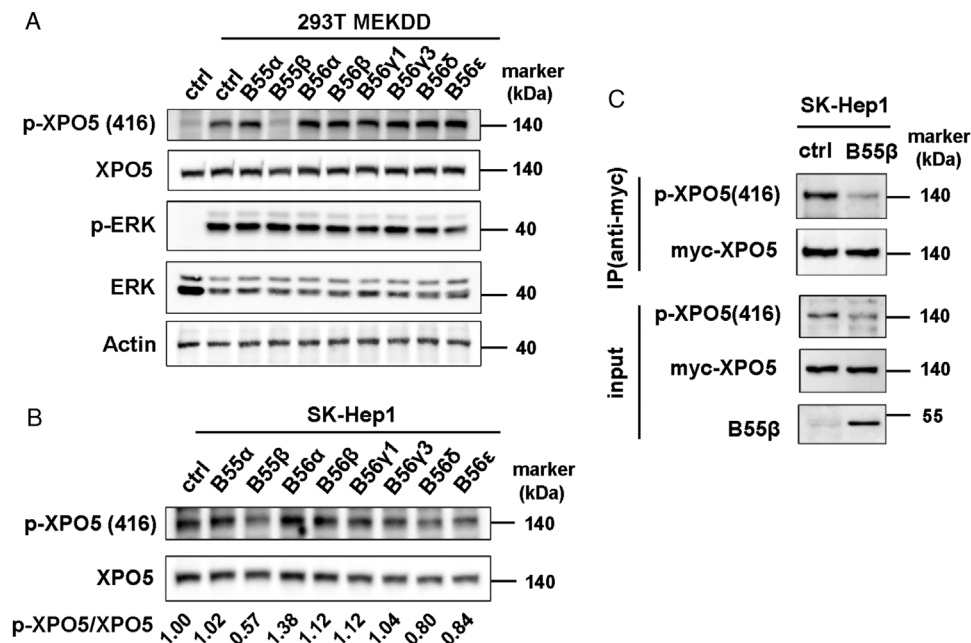


FIGURE 2 B55β is specifically engaged in XPO5 dephosphorylation. (A) HEK-293T cells were transfected with plasmids expressing MEKDD and different regulatory subunits. The phosphorylated and total XPO5/ERK proteins were detected via Western blot. (B) SK-Hep1 cells were transfected with plasmids expressing different regulatory subunits. The phosphorylated and total XPO5 proteins were detected via Western blot and the protein bands were quantified by ImageJ software. Comparison of the intensity of p-XPO5 bands (using total XPO5 as loading control) was indicated as fold change relative to ctrl set. (C) SK-Hep1 cells were co-transfected with the indicated plasmids and myc-XPO5 was immunoprecipitated from cell lysates. The immunoprecipitates were immunoblotted with the indicated antibodies.

further validation in HCC, we also screened these regulatory subunits in HCC SK-Hep1 cells. Consistently, overexpression of B55β promoted the dephosphorylation of XPO5 (Figure 2B). Moreover, when XPO5 was immunoprecipi-

tated in SK-Hep1 cells following the transfection of B55β, we observed a marked reduction of p-XPO5 (Figure 2C), supporting the engagement of B55β regulatory subunit in XPO5 dephosphorylation.

2.3 | B55 β reduces the proliferation, migration, and invasion abilities of HCC cells

Compelling evidence highlights that PP2A is functionally inactivated in cancers, typically through loss of heterozygosity and/or aberrant expression of PP2A subunits.²⁸ Previous studies reported that the B55 β -coding gene, *PPP2R2B*, was epigenetically silenced due to the upregulation of the histone methyltransferase *EZH2* in HCC.²⁹ To verify the expression of B55 β in HCC, paired tumorous samples and their adjacent normal tissues were collected and analyzed. The results showed that B55 β was dramatically reduced in HCC tissues (Figure 3A), suggesting its tumor-suppressive role in HCC progression. Because SK-Hep1 and Huh-7 HCC cell lines show the highest and the lowest p-ERK/p-XPO5 levels among the HCC cell lines we examined, respectively,¹⁶ they were chosen to investigate B55 β function. Based on the decreased expression of B55 β in HCC, we established the stable cell line overexpressing B55 β and scramble vector in SK-Hep1 cells (SK-Hep1 B55 β and SK-Hep1 ctrl). SK-Hep1 B55 β cells exhibited a lower p-XPO5 level as well as a decreased proliferation ability compared to the control cells (Figure 3B–D). Accordingly, B55 β overexpression remarkably suppressed cell migration and invasion in SK-Hep1 cells (Figure 3E and F).

To further determine the phenotype of B55 β in HCC, Huh-7 stable cell line overexpressing MEKDD (Huh-7 MEKDD) were established to trigger ERK/XPO5 signaling (Figure 3G), along with the promoted cell proliferation ability (Figure 3H and I). However, subsequent upregulation of B55 β decreased p-XPO5 level and rescued cellular phenotypes (Figure 3G–I), implying that B55 β might exert its antitumor activity via antagonizing ERK-induced XPO5 phosphorylation. Moreover, we observed a reversible transformation of cellular morphology in these transfectants (Supplementary Figure S1), supporting the regulatory role of B55 β in cell movement. To test this, transwell migration and wound healing assays were performed. As expected, B55 β abrogated ERK-induced cell migration (Figure 3J and K). Collectively, B55 β exerts a tumor-suppressive role in HCC at least in part by counteracting ERK-driven signals.

2.4 | B55 β regulates miRNA expression in HCC cells

Since XPO5 primarily shuttles between the nucleus and the cytoplasm to transport pre-miRNAs and our previous findings indicated that ERK-induced XPO5 phosphorylation trapped XPO5 in the nucleus,¹⁵ we next examined whether B55 β -mediated dephosphorylation could impinge on the

subcellular distribution of XPO5. As indicated in Figure 4A and Supplementary Figure S2A, XPO5 was spread in both the nucleus and the cytoplasm of Huh-7 cells with low XPO5 phosphorylation. Increased XPO5 phosphorylation by ERK activation kept XPO5 in the nucleus, whereas B55 β overexpression recovered the cytoplasmic localization of XPO5 (Figure 4A and Supplementary Figure S2A). Analogously, SK-Hep1 B55 β cells with lower p-XPO5 showed more cytoplasmic distribution of XPO5 when compared with the control cells (Figure 4B and Supplementary Figure S2B). These findings indicate the involvement of B55 β in modulating the cellular distribution of XPO5.

Given that XPO5-mediated nuclear export is important for miRNA maturation, we explored whether B55 β -catalyzed XPO5 dephosphorylation affected miRNA expression. We previously demonstrated that ERK-triggered XPO5 phosphorylation, coupled with subsequent conformation change, hindered the expression of miR-122 and miR-200b in HCC.^{15,16} miR-122 is a hepatocyte-specific miRNA accounting for about 70% of total liver miRNAs and exerts a dominant role in liver homeostasis and hepatocarcinogenesis.^{30–32} Huh-7 cells resemble normal hepatocytes in high abundance of miR-122.³³ In accord with the alternation in XPO5 compartmentalization (Figure 4A), ERK-triggered XPO5 phosphorylation impeded miR-122 expression, which was rescued by B55 β augmentation (Figure 4C). The similar results were observed in SK-Hep1 cells (Figure 4D). Additionally, miR-200b, a frequently downregulated tumor suppressor in a variety of tumors, was also elevated by B55 β -induced XPO5 dephosphorylation (Figure 4D). Taken together, the expression of these tumor-suppressive miRNAs is negatively correlated with the level of XPO5 phosphorylation, and the regulatory B55 β subunit could antagonize the effect of ERK kinase on XPO5, thereby promoting the expression of these miRNAs.

2.5 | B55 β inhibits cell proliferation and migration through modulating miRNA expression

Based on the above findings that the regulatory B55 β subunit modulates miRNA expression, we further investigated whether B55 β exerted its biological significance through affecting these miRNAs. miR-200b is illustrated to inhibit tumor metastasis by regulating epithelial-mesenchymal transition (EMT).^{34,35} To provide evidence for the engagement of miR-200b in B55 β -mediated anti-HCC program, SK-Hep1 B55 β cells were transfected with anti-miR-200b to lower its expression (Figure 5A). As indicated in Figure 5B and C, B55 β -mediated suppression of cell migration was reversed by the subsequent inhibition of miR-200b,

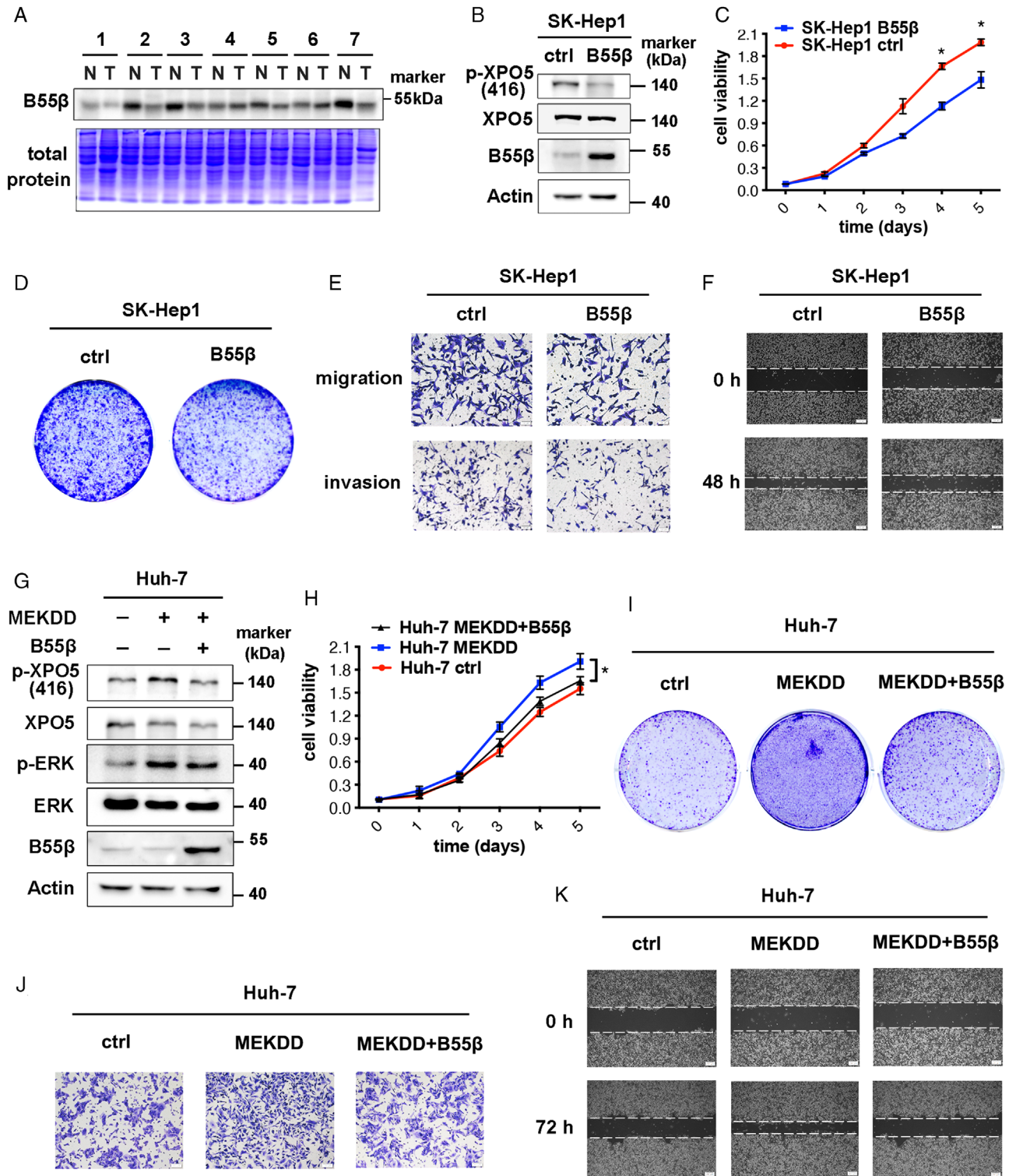


FIGURE 3 B55β inhibits growth, migration and invasion abilities of HCC cells. (A) The protein lysates of human HCC and adjacent normal tissues were subjected to SDS-PAGE and then stained with coomassie blue or immunoblotted with anti-B55β antibody. (B) B55β level was determined by Western blot in SK-Hep1 stable cell lines with or without B55β overexpression. (C–F) MTT assays (C), cloning formation (D), transwell migration and invasion assays (E), and wound healing (F) for SK-Hep1 B55β overexpression and ctrl cells. (G) B55β level was detected by Western blot in Huh-7 stable cell lines with or without MEKDD/B55β overexpression. (H–K) MTT assays (H), cloning formation (I), transwell migration assays (J), and wound healing (K) for Huh-7 stable cell lines with or without MEKDD/B55β overexpression. Data are shown as the means ± SD. **p* < 0.05.

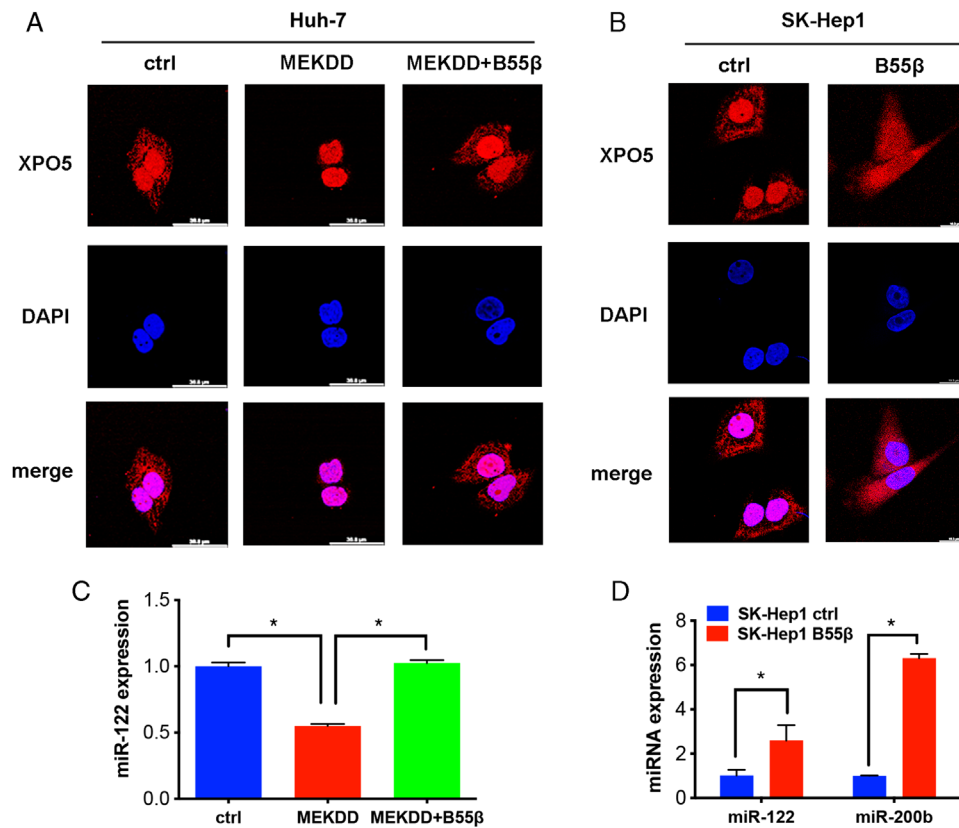


FIGURE 4 B55 β regulates XPO5 distribution and miRNA expression. (A) Immunofluorescence of subcellular localization of XPO5 (Red) in Huh-7 cells transfected with the indicated plasmids. (B) Immunofluorescence of subcellular localization XPO5 (Red) in SK-Hep1 Ctrl and B55 β overexpression cells. (C) The miR-122 expression in Huh-7 cells transfected with the indicated plasmids was determined by qRT-PCR. (D) The expression of miR-122 and miR-200b in SK-Hep1 ctrl and B55 β overexpression cells was examined by qRT-PCR. Data are shown as the means \pm SD. * $p < 0.05$, ** $p < 0.01$.

indicating that the antimigratory activity of B55 β is achieved in part by upregulating miR-200b. Similarly, the antiproliferative effect of B55 β was weakened upon the inhibition of miR-122 expression (Figure 5D–F), implying the active involvement of miR-122 in B55 β -mediated regulatory network. Therefore, B55 β -mediated HCC inhibition is at least in part through elevating these tumor-suppressive miRNAs.

2.6 | B55 β suppresses HCC development in vivo

We next evaluated the in vivo function of B55 β in the HCC xenograft model. In line with the above findings, B55 β significantly inhibited tumor growth when compared with control set (Figure 6A and B). Importantly, p-XPO5 was downregulated, whereas miR-122 and miR-200b were elevated in SK-Hep1 B55 β tumor (Figure 6C and D), indicating that B55 β exerts in vivo anti-HCC role through dephosphorylating XPO5 and promoting some miRNA expression.

3 | DISCUSSION

The spatial and temporal expression of miRNAs are key for cell lineage decisions and tissue homeostasis, while the overall downregulation of miRNA is observed in HCC and causally involved in tumor development.^{3–5} Increasing evidence supports the defect in miRNA biogenesis drives miRNA dysregulation and subsequent oncogenic transformation of various cell types.^{4,5} Our previous work showed that ERK-induced phosphorylation of XPO5, followed by Pin1-mediated isomerization, hindered miRNA biogenesis in HCC.^{15–18} However, little is informed about the process of XPO5 dephosphorylation. In this study, we revealed that the serine/threonine phosphatase PP2A specifically catalyzed the dephosphorylation of XPO5, while this modulatory mechanism was impaired due to PP2A downregulation in HCC (Figure 7). Further augmentation of PP2A restored the cytoplasmic distribution of XPO5 and promoted the expression of several tumor-suppressive miRNAs. Thus, our study provides new insight into miRNA dysregulation in HCC.

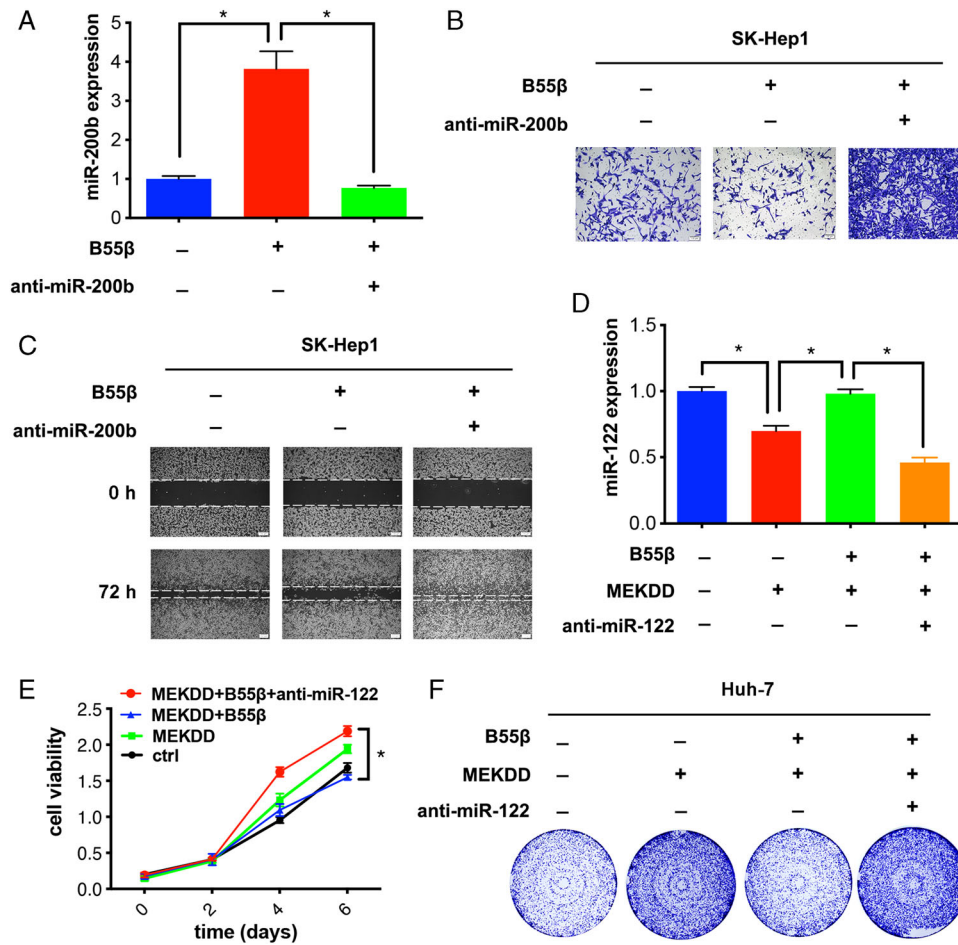


FIGURE 5 B55β-induced HCC inhibition is partially attributed to the regulation of miRNA expression. (A) SK-Hep1 ctrl and B55β overexpression cells were transfected with or without anti-miR-200b, followed by the examination of miR-200b expression via qRT-PCR. (B) Transwell migration assays and (C) wound healing assays for SK-Hep1 stable cell lines transfected with or anti-miR-200b. (D) miR-122 expression was detected by qRT-PCR in Huh-7 cells transfected with or without anti-miR-122. (E) MTT assays and (F) cloning formation assays for Huh-7 stable cell lines transfected with or without anti-miR-122. Data are shown as the means ± SD. **p* < 0.05.

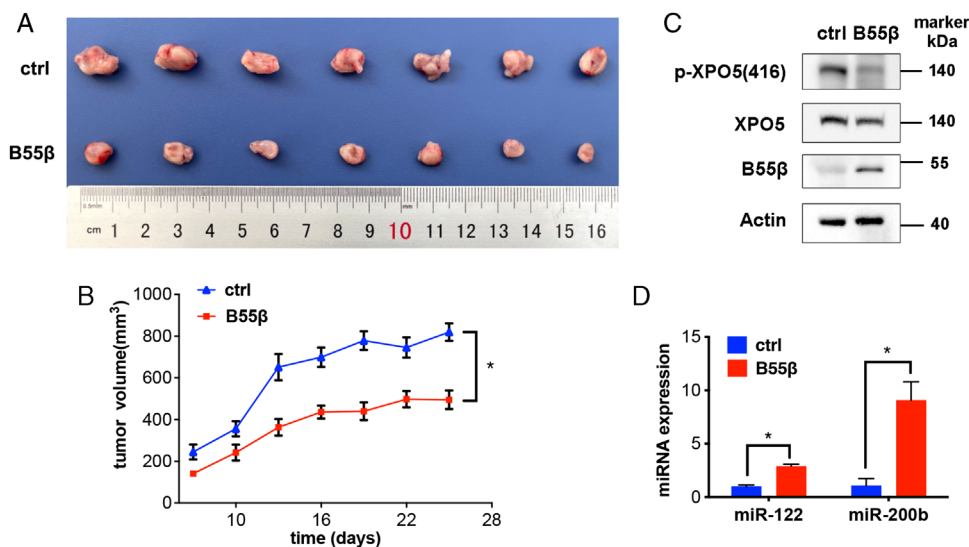


FIGURE 6 B55β represses HCC progression via tuning miRNA expression in vivo. (A) Photograph and (B) tumor growth curve of SK-Hep1 ctrl and B55β overexpression xenografts in nude mice. (C) Phosphorylated and total XPO5 as well as B55β in xenografts was detected by Western blot. (D) miRNA expression in xenografts was measured by qRT-PCR. Data are shown as the means ± SD. **p* < 0.05, ***p* < 0.01.

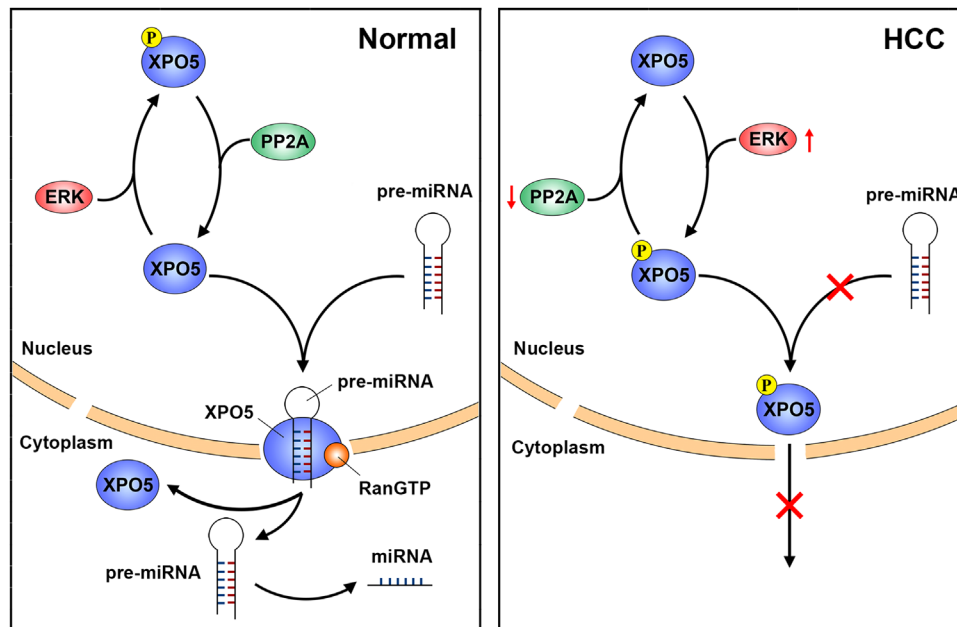


FIGURE 7 Schematic diagram of how PP2A modulates miRNA expression. In normal conditions, phosphatase PP2A dephosphorylates XPO5 and modulating the transport ability of XPO5, thus promoting miRNA expression. In HCC, PP2A downregulation coupled with ERK activation favors XPO5 phosphorylation, leading to the compromised miRNA expression.

The PP2A holoenzyme is a ternary complex consisting of a catalytic subunit, a scaffold subunit, as well as a regulatory subunit responsible for recruiting substrates. Among the available PP2A regulatory subunits in hand, we identified that the B55 β subunit remarkably reduced the level of p-XPO5 in both HEK-293T and HCC cell lines (Figure 2A and B), and further confirmed the involvement of B55 β in XPO5 dephosphorylation via immunoprecipitation of XPO5 upon B55 augmentation and examination of the phosphorylated status of the recovered protein (Figure 2C). In view of the diversity of the PP2A regulatory subunit family, we can't rule out the possibility that other regulatory subunits are also engaged in the regulation of XPO5 phosphorylation.

PP2A primarily functions as a tumor suppressor through tuning the phosphorylation status and the function of important cancer-associated proteins, such as c-Myc and p53.^{19,36,37} Our study identified XPO5 as a potential substrate for B55 β -containing PP2A and further clarified that B55 β -containing PP2A can inhibit HCC progression by promoting XPO5-dependent maturation of several tumor-suppressive miRNAs, providing another potential mechanism to interpret the anti-tumor role of PP2A. Cancer cells employ various strategies to evade PP2A-mediated tumor suppression, such as dysregulation of PP2A subunits and its binding partners, or loss of phosphatase activity.^{38–41} For example, *PPP2R2A*, the α isoform of B55 regulatory subunit family, is frequently deleted in luminal B breast cancers, which adds a significant pathophysiological feature to

this cancer subtype.³⁹ In this study, we examined expression of B55 β subunit in paired HCC and adjacent normal samples and showed the general downregulation of B55 β in HCC tissues (Figure 3A), supporting it as a potential target for HCC therapy. Currently, pharmacological strategies for targeting PP2A in human cancer are developed. For example, small molecules are designed to selectively promote the assembly of the holoenzyme with specific subunits.^{42–44} These findings instill promise for restoring the function of B55 β -containing PP2A in HCC in a selective manner.

Our findings found that PP2A-mediated dephosphorylation of XPO5 restored the biogenesis of several important tumor-suppressive miRNAs (e.g., miR-122 and miR-200b) via antagonizing ERK kinase (Figure 4). MiR-122, the most abundant miRNA in normal liver, is drastically downregulated in HCC.⁴⁵ Deletion of the *miR-122* gene leads to hepatocarcinogenesis in mice.^{31,32} MiR-200b is another key tumor-suppressive miRNA that inhibits tumor metastasis through regulating various proteins, such as ZEB and moesin.^{34,35,46} However, it is clear that not all miRNA is governed by PP2A/XPO5 axis. In addition to the canonical miRNA biogenesis, recent studies have characterized the alternative pathways for miRNA processing.^{6,47} During cellular quiescence, a subset of pri-miRNAs with a 2,2,7-trimethylguanosine (TMG)-cap is induced and processed in a XPO5-independent but XPO1-dependant manner.⁴⁷ Therefore, miRNA biogenesis is much more complex than previously supposed

and further work is needed to illustrate the detailed network.

4 | MATERIALS AND METHODS

4.1 | Reagents

Anti-XPO5, anti-actin, anti-PP2A catalytic subunit, anti-p-ERK, anti-ERK, and anti-myc tag antibodies for Western blot were from Cell Signaling Technology (Danvers, MA, USA). Anti-B55 β antibody for Western blot was purchased from Proteintech (Wuhan, China). Anti-XPO5 antibody for immunofluorescence was from Novus Biologicals (USA). Antibodies to the Ser416 phosphorylation site of XPO5 were generated in collaboration with Lifetein LLC. Anti-c-Myc agarose beads were from Sigma-Aldrich (St. Louis, MO, USA). Anti-miRNA oligos were purchased from GenePharma Company.

4.2 | Plasmids

The plasmids B55 α (#13804), B55 β (#16181), B56 α (#14532), B56 β (#14533), B56 γ 1 (#14534), B56 γ 3 (#14535), B56 δ (#14536), and B56 ϵ (14537) were bought from Addgene. The full length of B55 β (#16181, Addgene) cDNA was subcloned into the pCDH-CMV-MCS-EF1-copGFP vector.

4.3 | Cell culture

SK-Hep1, Huh-7, and HEK-293T cells were cultured in DMEM medium with 10% fetal bovine serum at 37°C in a 5% CO₂ incubator.

4.4 | Cell transfection

For transient expression, cells were transfected with plasmids using Lipofectamine 2000 transfection reagent. Briefly, cells were seeded into 6-well plates at a density of 3×10^5 cells per well and incubated overnight prior to transfection. Plasmids and Lipofectamine 2000 were diluted separately in Opti-MEM medium (Invitrogen) and left for 5 min at room temperature. The diluted DNA and Lipofectamine 2000 were then gently mixed together at a ratio of 1 μ g DNA/2.5 μ l Lipofectamine 2000 and incubated for 20 min at room temperature. Finally, the mixture was added to cells and incubated for a certain time before performing subsequent experiments. The medium was changed after 6 h. For lentivirus preparation, HEK-293T cells were co-transfected with pCDH-CMV-MCS-EF1-copGFP plasmids expressing B55 β , the packaging plasmid pCMV-dR8.2

dvpr and the envelope plasmid pCMV-VSVG (System Bioscience). SK-Hep1 and Huh-7 cells were infected with the resultant lentivirus and stable cell lines were obtained after GFP sorting.

4.5 | Clinical samples

Primary HCC tumor tissues and the adjacent normal tissues were obtained from West China Hospital with written informed consent for research purpose. All procedures were approved by the Ethics Committee of West China Hospital of Sichuan University. For total protein preparation, the patient tissues were cut up and grounded into powder in liquid nitrogen. Subsequently, RIPA buffer was added and sonicated, followed by centrifugation at 12,000 rpm for 15 min at 4°C. The resultant supernatants were collected for Western blot analysis.

4.6 | Western blot

Total proteins were extracted with RIPA buffer (25 mM Tris-HCl, pH 7.5, 0.1% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, 150 mM NaCl) supplemented with phosphatase inhibitor and protease inhibitor cocktail and quantified with the BCA assay. Equal amounts of proteins were added to each well of SDS-PAGE gels and transferred to PVDF membranes (Millipore). After blocking with 5% non-fat milk or BSA for 1 h at room temperature, membranes were incubated with primary antibody at 4°C overnight and secondary antibody at room temperature for 1 h. The chemiluminescence signals were detected using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific).

4.7 | MTT assay

Cells were seeded into 96-well plates at a density of 3×10^3 cells per well and cultured for a certain time. At the indicated time points, 10 μ l of MTT (5 mg/ml, Sigma) was added into each well and incubated at 37°C for 3 h. After the supernatant was removed, the resultant purple formazan crystals were dissolved in DMSO. The absorbance was measured at a wavelength of 570nm by UV spectrophotometer (Thermal Fisher).

4.8 | Cloning formation assay

Cells were seeded into 6-well plates at a density of 3×10^3 cells per well and cultured for several days. The plates were

then fixed by 4% paraformaldehyde (PFA) for 20 min and stained with 1% crystal violet for 15 min.

4.9 | Transwell migration and invasion assay

Transwell chambers (8.0 μm) with or without Matrigel coating were used for cell migration and invasion assays. For migration assay, 1×10^5 cells were suspended in 200 μl of serum-free medium and seeded into the top chambers in the 24-well plate, while medium with 10% FBS was added into the bottom chambers. For invasion assay, the chamber was precoated with Matrigel (BD Biosciences) and 2×10^5 cells were seeded in each chamber. After 24 h incubation, cells in the top chambers were fixed with 4% PFA for 20 min and stained with 1% crystal violet for 15 min.

4.10 | Wound healing assays

For wound healing assays, cells were cultured in 6-well plates to full confluency. The sterilized pipet tips were used to scratch the monolayer cells. After washing three times with PBS, cells were cultured in DMEM medium with 0.5% FBS. Images were taken using the microscope after 48 or 72 h.

4.11 | Immunoprecipitation assay

The immunoprecipitation assay was performed as previously described.¹⁶ Briefly, cells were co-transfected with plasmids expressing myc-XPO5 and B55 β . After 48 h of transfection, cells were collected and lysed with cold lysis buffer (0.1% NP-40, 100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, and protease and phosphatase inhibitor cocktail). After incubation on ice for 15 min, cells were homogenized for 30 strokes. The lysates were then centrifugated at $2000 \times g$ for 15 min and subjected to immunoprecipitation with anti-myc agarose beads (Sigma). Immunoprecipitates were washed three times with washing buffer (0.5% NP-40, 150 mM NaCl, 0.5 mM EDTA, 20 mM Tris-HCl, pH 7.5). The bound proteins were eluted with SDS-PAGE loading buffer for Western blot.

4.12 | Immunofluorescence analysis

Huh-7 or SK-Hep1 stable transfectants were seeded on the glass cover slides and incubated overnight. Then cells were fixed with 4% PFA, permeabilized with 0.5% Triton X-100, blocked with 7% BSA, and incubated with anti-XPO5 anti-

body at 4°C overnight. After washing with PBS three times, cells were incubated with Alexa Fluor 647 dye-conjugated secondary antibodies at room temperature for 1 h and stained with DAPI solution for 10 min at room temperature. Confocal fluorescence images were acquired using Leica STELLARIS 5 confocal spectral microscope.

4.13 | miRNA quantification

TRIzol reagent (Invitrogen) was used to extract total RNAs from cells, and M-MLV Reverse Transcriptase Kit (Invitrogen) was used to generate cDNAs according to the manufacturer's instructions. qRT-PCR was performed to examine the levels of mature miRNAs by SYBR Green Master Mix Kit (Applied Biosystems) and QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems). U6 was used as the internal control. Primers specific for miRNA quantification were ordered from GenePharma.

4.14 | Animal experiment

All the procedures in mice experiments were approved by the Ethics Committee of West China Hospital of Sichuan University. Mice were randomly assigned to experimental groups. SK-Hep1 stable cell lines were collected and suspended at $5 \times 10^6/\text{ml}$ in saline solution with 50% Matrigel (Corning). Then 100 μl of cells were subcutaneously injected into BALB/c nude mice. The length and width of xenografts were measured every 3 days with calipers. Tumor volume was calculated using the formula: volume (mm^3) = $ab^2/2$ (a , long diameter; b , short diameter). Proteins and RNAs in xenografts were extracted for B55 β as well as phosphorylated and total XPO5 detection.

4.15 | Statistical analysis

The experimental data were represented as the mean \pm SD of at least three biological replicates. Two-tailed Student's t test was used to determine the statistical significance of parametric data. Statistical tests were analyzed using GraphPad Prism software. p Values less than 0.05 was considered statistically significant.

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CONFLICT OF INTEREST

The author Yong Peng is an associate editor of *MedComm* and not involved in the journal's review of, or decisions

related to, this manuscript. The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Jiao Li and Jian-Kang Zhou performed cell biology, biochemistry, and animal experiments; Xiaoyu Mu helped to construct the plasmids; Shu Shen and Xiaomin Xu provided patient samples; Yao Luo and Yuxin Luo helped to perform immunofluorescence assay; Yue Ming and Yuan-gang Wu helped to establish xenograft models. Jiao Li, Jian-Kang Zhou and Yong Peng wrote the manuscript; Yong Peng oversaw the experimental design and data analysis.

ETHICS APPROVAL

All animal and patient sample studies were approved by the Ethics Committee of West China Hospital of Sichuan University. Written informed consent for research purposes was obtained from the patients.

DATA AVAILABILITY STATEMENT

All data are available from the corresponding authors upon request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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